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MULTIPLE MECHANISMS MEDIATE GLUCOSE REGULATION OF *MAL* GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

by

ZHEN HU

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

MULTIPLE MECHANISMS MEDIATE GLUCOSE REGULATION OF *MAL* GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

by

Zhen Hu

Advisor: Professor Corinne A. Michels

Maltose fermentation in *Saccharomyces cerevisiae* requires at least one of five unlinked *MAL* loci. Each *MAL* locus is a complex of three genes: Gene 1 encodes maltose permease; Gene 2 encodes maltase; Gene 3 encodes the *MAL*-activator. Expression of the *MAL* structural genes is induced by maltose and repressed by glucose. Maltose induction is mediated by a *MAL*-activator and requires the presence of a functional maltose permease gene. Two *MAL* structural genes share a bidirectional promoter which contains the upstream activating sequence, the $\mathrm{UAS}_{\mathrm{MAL}}$. We undertook the present study to analyze the mechanisms of glucose repression of *MAL* gene expression.

MIG1 encodes a zinc-finger DNA binding protein which represses the transcription of *SUC2* and *GAL* genes in response to glucose. Mig1p is a downstream component of the Snf1p kinase signal transduction pathway that mediates glucose repression. We demonstrate that Mig1p binds to two sites located in the *MAL61-MAL62* intergenic region and represses the transcription of these two *MAL* structural genes in the presence of glucose. Mig1p also binds to the promoter of *MAL63* and represses the transcription of this *MAL*-activator gene. Studies using constitutive *MAL*-activator alleles reveal that glucose inhibits the *MAL*-activator-mediated maltose induction by mechanisms that are independent of Mig1p.

HXK2 encodes a glucose phosphorylating enzyme, hexokinase PII. We found that Hxk2p mediates glucose repression of maltase expression including those repression effects that are independent of Mig1p. *HXK1* encoding hexokinase PI, in *hxk2* background, is able to play some of the roles of Hxk2p in transmitting/generating glucose repression signal acting on the *MAL* promoters.

We show that overexpression of the *MAL*-activator is not sufficient to relieve glucose inhibition of maltose induction. Moreover, elimination of inducer exclusion does not alleviate glucose inhibition of the function of the overexpressed *MAL*-activator. These results suggest that glucose inhibits maltose induction by affecting the induction process and/or events required for induction (induction-dependent mechanism). This mechanism requires *REG1* (a regulatory subunit of protein phosphatase type-1), *GRR1* (a putative ubiquitin protein ligase), as well as *HXK2*, but does not appear to involve *RGT1* (a repressor of hexose transporter genes) and *RGT2* (a high-glucose sensor).

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INTRODUCTION

Part I. Glucose Repression in *Saccharomyces cerevisiae*

In the yeast *Saccharomyces cerevisiae*, growth on glucose results in the repression of a large number of genes required for the utilization of alternative carbon sources, such as sucrose, galactose, maltose, glycerol, ethanol, acetate, as well as gluconeogenesis, the TCA cycle, respiration, and mitochondrial function (Figure 1) (for reviews see Johnston and Carlson, 1992; Trumbly, 1992; Gancedo, 1992; Ronne, 1995). This regulatory phenomenon, termed glucose repression, allows yeast cells to utilize the most effectively metabolized carbon source, glucose, when dealing with multiple carbon sources present in the environment. The mechanism of glucose repression has been under intensive investigation for years but is still poorly understood. The regulatory pathway seems to involve multiple steps and branches operating on subsets of glucose-repressible genes. In most cases, glucose control acts mainly at the transcriptional level (Johnston and Carlson, 1992; Trumbly, 1992; Gacendo 1992). A multitude of genes involved in either glucose repression or derepression in the absence of glucose have been identified by various genetic methods (Trumbly, 1992; Gacendo, 1992). Mutations in some of these genes were isolated in searches for glucose-resistant mutants affecting *SUC2*, *GAL*, and *MAL* gene regulation. Others were isolated by suppressor analysis. A few of

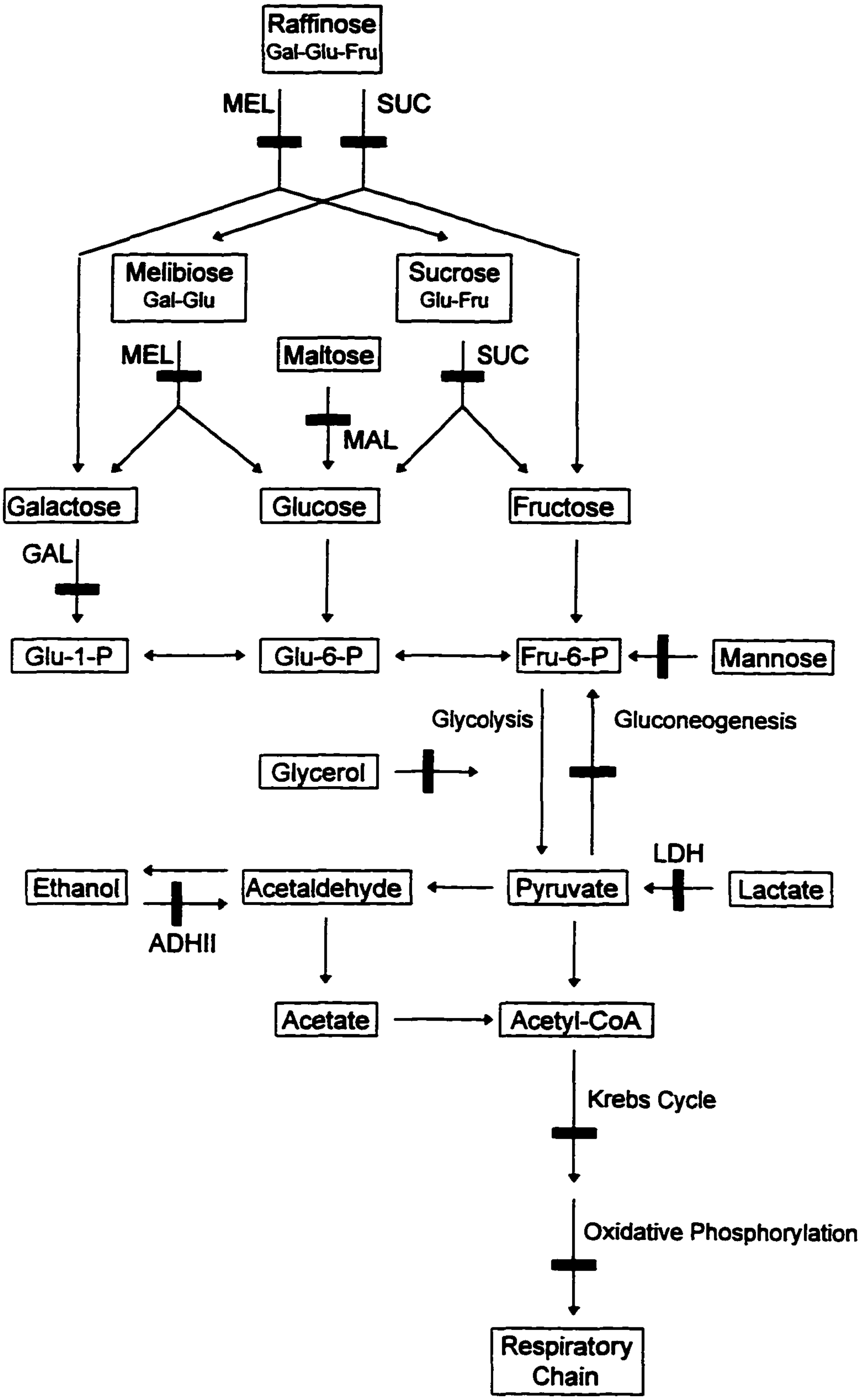


Figure 1. Schematic representation of the glucose-repressed steps (black bars) in carbohydrate and energy metabolism.

these genes were identified in two-hybrid screens (Fields and Song, 1989) for proteins capable of interaction with known factors required for repression or depression. In the following sections I will discuss the functions of those crucial genes, their roles in glucose repression, and, finally, the target genes that are subject to glucose repression.

Genes affecting hexose transporter expression

S. cerevisiae contains an *HXT* multigene family of glucose transporters (Bisson *et al.*, 1993). Members of yeast sugar transporter superfamily typically contains 12 putative membrane-spanning domains. Glucose-induced expression of *HXT* genes exhibits at least three different patterns (Ozcan and Johnston, 1995): *HXT3* is induced by glucose independent of the sugar concentration; *HXT1* is induced only by high concentrations of glucose; *HXT2* and *HXT4* are induced by low levels of glucose and repressed at high concentrations of glucose. *SNF3* encodes an 884 amino acid protein that resembles other glucose transporters (Celenza *et al.*, 1988) and is required for high affinity glucose transport (Bisson *et al.*, 1987). However, Snf3p possess an unusually long carboxyl-terminal domain which is necessary for Snf3p function (Coons *et al.*, 1997). Overexpression of this C-terminal domain is able to complement the growth defect on low glucose caused by *snf3* null mutation (Coons *et al.*, 1997). This, along with the low level of *SNF3* expression strongly suggests a regulatory role of Snf3p (Marshall-Carlson, 1990; Coons *et al.*, 1997). Mutations in *RGT1* and

RGT2 genes were originally isolated that suppressed the high-affinity glucose transport defect associated with a *snf3Δ* mutant (Marshall-Carlson *et al.* 1991).

GRR1 encodes a 132-kD protein containing two important domains, the F-box and leucine-rich repeats, both of which are proposed to mediate protein-protein interaction (Flick and Johnston, 1991; Li and Johnston, 1997). *GRR1* is constitutively expressed at low levels (Flick and Johnston, 1991). Mutations in *GRR1* relieve glucose repression of many glucose-repressible genes (Bailey and Woodword, 1884) and cause a variety of pleiotropic defects (Flick and Johnston, 1991; Conklin *et al.*, 1993). The slow growth of *grr1* mutants on glucose led to the discovery that *grr1* mutant cells are deficient in glucose uptake (Vallier *et al.*, 1994; Ozcan *et al.* 1994) and defective in glucose induction of *HXT1-4* expression (Ozcan and Johnston, 1995). The *rgt1* mutations suppress the glucose growth defect of *grr1* mutations and restore both high-affinity glucose transport and glucose repression in *grr1* mutant strains (Vallier *et al.*, 1994; Erickson and Johnston 1994). *RGT1* encodes a zinc cluster DNA-binding protein which represses transcription of *HXT* genes (Ozcan and Johnston, 1996; Ozcan *et al.*, 1996). *RGT1* is constitutively expressed at low levels. Grr1p is a negative regulator of Rgt1p in response to glucose (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996). Thus, glucose induces *HXT* expression by activating Grr1p which in turn inhibits the function of the Rgt1p repressor. Relief of glucose repression is associated with high-affinity glucose transport defect in *grr1* mutants (Vallier *et* *al.*, 1994). Repression of *HXT* genes by Rgt1p in the absence of glucose depends on *SSN6* and *TUP1* genes (Ozcan *et al.*, 1996).

The dominant *RGT2-1* mutation restores growth of a *snf3Δ* on low glucose and causes constitutive expression of *HXT* genes (Ozcan *et al.*, 1996). *RGT2* encodes a putative glucose transporter highly homologous to Snf3p and is required for high glucose-induced expression of *HXT1* gene (Ozcan *et al.*, 1996). *HXT* gene expression activated by *RGT2-1* depends on the function of Grr1p. *SNF3* is required for low glucose-induced expression of *HXT2* and *HXT4*. Expression of *SNF3* is repressed by high levels of glucose (Celenza *et al.*, 1988; Ozcan *et al.*, 1996). Taken together, these results suggest that Snf3p and Rgt2p are sensors of low and high extracellular glucose, respectively, and are involved in generating a glucose signal required to induce *HXT* expression via Grr1p (Ozcan *et al.*, 1996).

Hexokinase PII

Mutations in *HXK2* encoding hexokinase PII were isolated as mutants resistant to glucose repression (Zimmermann and Scheel, 1977; Entian and Zimmermann, 1980; Michels and Romanowski, 1980). The mechanism by which Hxk2p affects glucose repression is unclear. *HXK2* is expressed on various carbon sources, and appears to be induced to a higher levels on glucose and fructose (Herrero *et al.*, 1995). Entian and colleagues suggested that Hxk2p has separate catalytic and regulatory domains (Entian and Frohlich, 1984; Entian *et al.*, 1985).

However, more recent studies suggest that the catalytic activity of both hexokinase PI and PII are involved in glucose repression (Ma *et al.*, 1989; Rose *et al.*, 1991). Hxk2p also has been reported to possess protein kinase activity that is regulated by glucose (Herrero *et al.*, 1989). Since Hxk2p phosphorylates glucose, it is likely that Hxk2p is involved in production and/or transduction of the signal for glucose availability.

There are two isozymes of hexokinase in yeast: hexokinase PI encoded by *HXK1* and hexokinase PII encoded by *HXK2* (Lobo and Maitra, 1977). Expression of *HXK1* is repressed by glucose and the maintenance of repression requires *HXK2* (Herrero *et al.*, 1995; De Winde *et al.*, 1996). Null mutations in *HXK1* have no effect on glucose repression (Ma and Botstein, 1986). However, multiple copies of *HXK1* partially restore glucose repression in an *hxk2Δ* mutant (Ma and Botstein, 1986; Rose *et al.*, 1991). Another glucose-phosphorylating enzyme, glucokinase encoded by *GLK1*, does not seem to play a role in glucose repression (Rose *et al.*, 1991). Glucose repression of *GLK1* itself also depends on *HXK2* (Herrero *et al.*, 1995; De Winde *et al.*, 1996).

REG1* and *GLC7

Mutations in *REG1* were recovered in a search for mutants able to grow on sucrose in the presence of 2-deoxyglucose, a glucose analog that cause glucose repression but can not be metabolized (Neigeborn and Carlson, 1987). The same selection also yielded *hxk2* and *cid1*. *REG1* encodes a protein of 1026 amino acids which appears to be a nuclear protein (Niederacher and Entian, 1991). Overexpression of *REG1* on a multiple copy plasmid significantly reduces *GAL* gene expression (Niederacher and Entian, 1991). *REG1* was also identified as a second site-suppressor of mutations in *RNA1* encoding a GTPase-activating protein (GAP) specific to Gsp1, the yeast homologue of the mammalian Ran/TC4 GTPase (Tung *et al.*, 1992; Becker *et al.*, 1995). A recent study also implicates *REG1* in glycogen accumulation process, though the effect of which appears to be indirect (Huang *et al.*, 1996).

The recessive mutation *cid1-226* relieves glucose repression of invertase (*SUC2*) and maltase (*MAL32*) expression but not *GAL1* (Neigeborn and Carlson, 1987). Cloned *CID1* was found to be allelic to *GLC7*, an essential gene encoding the catalytic subunit of protein phosphatase type I (PP1) (Tu and Carlson, 1994). PP1 is highly conserved between yeast and mammals (Ohkura *et al.*, 1989; Feng *et al.*, 1991). Like its mammalian counterparts, the yeast PP1 participates in various physiological pathways ranging from glycogen accumulation and sporulation (Feng *et al.*, 1991; Cannon *et al.*, 1994) to cell cycle control (Francisco *et al.*, 1994; Hisamoto *et al.*, 1994; Zhang *et al.*, 1995) and protein translation (Wek *et al.*, 1992) as well as glucose repression. *GLC7* appears to function antagonistically to Snf1 protein kinase, which is required for release from glucose repression, since the effect of the *glc7-T152K* (*cid1-226*) mutation is suppressed by mutations in *SNF1* (Tu and Carlson, 1994). The *glc7-T152K* mutant does not impair glycogen accumulation while the *glc7-1* mutant is defective in glycogen accumulation and sporulation but does not affect glucose repression (Cannon *et al.*, 1994; Tu and Carlson, 1994). PP1 exhibits little substrate specificity *in vitro*. It has been proposed that specific regulatory or targeting subunits direct the PP1 catalytic subunit to its site of activity and/or regulate its substrate specificity and regulatory properties (Cohen, 1989; Hubbard and Cohen, 1993).

Recent studies suggest that Reg1p is a regulatory subunit of PP1 (Tu and Carlson, 1995). Reg1 protein is physically and functionally associated with PP1. Overexpression of *LexA-REG1* not only reduces derepression of *SUC2* significantly but also suppresses the glucose repression defect of *glc7-T152K* (Tu and Carlson, 1995). The *glc7-T152K* mutation decreases interaction of Glc7p with Reg1p, but not with Gac1p, which is a glycogen regulatory subunit for PP1, and shows a defect in interaction with the Glc7-1 mutant protein (Stuart *et al.*, 1994; Tu and Carlson, 1995). Reg1p may regulate PP1 by targeting its activity to substrates involved in the glucose repression regulatory pathway.

Snf1 protein kinase complex

SNF1 was originally identified as a key component of the glucose repression regulatory pathway (Celenza and Carlson, 1986). It is now known that Snf1 protein kinase is conserved in eukaryotes (Jiang and Carlson, 1996). Mutations in *SNF1* result in growth defects on all carbon sources except glucose. *SNF1* has been found to be required for a variety of cellular processes (Celenza and

Carlson, 1986; Schuller and Entian, 1987; Thompson-Jaeger *et al.*, 1991; Simon *et al.*, 1992). The *snf1* mutants fail to derepress all of the glucose-repressible genes (reviewed in Johnston and Carlson, 1992). *SNF1* encodes a serine/threonine protein kinase and its expression is not glucose-repressible (Celenza and Carlson,1984a,b). The protein kinase domain has been shown to be essential for Snf1p function (Celenza and Carlson, 1989). Snf1p can be autophosphorylated *in vitro* (Celenza and Carlson, 1986).

Mutations in *SNF4* exhibit the same pleiotropic defects as *snf1* mutants (Neigeborn and Carlson, 1984; Schuller and Entian, 1987). *SNF4* encodes a 322 amino acid protein and is expressed constitutively (Schuller and Entian, 1987, 1988; Celenza *et al.*, 1989). Mutations in both *SNF1* and *SNF4* are suppressed by *ssn6* mutations (Carlson *et al.*, 1984; Neighborn and Carlson, 1984). The Snf4 protein is physically associated with Snf1p and is required for maximal kinase activity *in vitro* (Celenza and Carlson, 1989; Celenza *et al.*, 1989)

The Snf1 kinase contains two domains: an amino-terminal kinase domain and a carboxyl-terminal regulatory domain. Snf4p interacts with the Snf1 regulatory domain only in glucose-deprived cells (Jiang and Carlson, 1996). The Snf1 kinase domain binds directly to the regulatory domain in high glucose. Snf4p and the Snf1 kinase domain interact with overlapping sites in the Snf1 regulatory domain. It has been proposed that the Snf1 regulatory domain autoinhibits the kinase domain in high glucose, and that Snf4p counteracts the autoinhibition by binding to the regulatory domain of Snf1p in low glucose (Jiang and Carlson, 1996)

SIP1 and *SIP2* were identified in a two-hybrid screen for proteins interacting with Snf1p (Yang *et al.*, 1992). Partially dominant mutations in *GAL83* relieve glucose repression dependent of *SNF1* (Matsumoto *et al.*, 1981; Erickson and Johnston, 1993). *SIP1* was also isolated as a multicopy suppressor of *gal83* and shown to be a negative regulator of *GAL* gene expression in response to glucose (Mylin *et al.*, 1994). These three proteins are homologous to each other and have been shown to interact with Snf1p (Yang *et al.*, 1994). The association of Sip1p, Sip2p and Gal83p with Snf1p is not regulated by glucose availability and is independent of Snf1 kinase activity and *SNF4*. A highly conserved domain of approximately 80 residues at the C-termini of these three proteins, the ASC domain, mediates interaction with the Snf1 complex. Sip1p, Sip2p, and Gal83p appear to form complexes with Snf1p alternatively and have distinct but related functions. It is proposed that Sip1p, Sip2p, and Gal83p act as adapters that direct Snf1 kinase towards specific targets (Yang *et al.*, 1994).

Recent studies indicate that the ASC domain of Sip1p, Sip2p, and Gal83p actually interacts with Snf4p independent of Snf1p (Jiang and Carlson, 1997). Another conserved region internal to three proteins, the KIS domain, binds to Snf1p independent of Snf4p. Both interactions occur in cells grown in high glucose media. A model has been proposed in which Sip1p, Sip2p, or Gal83p serves as an anchor holding Snf1p and Snf4p together within the Snf1 kinase complex where the direct interaction between Snf1p and Snf4p is regulated by glucose availability (Jiang and Carlson, 1997).

The mammalian homologue of Snf1p is the catalytic α subunit of the AMP-activated protein kinase (AMPK) (Mitchelhill *et al.*, 1994; Carling *et al.*, 1994). AMPK consists of three subunits, α, β, and γ subunit. The Sip1p homologue, AMPKβ, contains regions corresponding to the KIS and ASC domains and has been shown to bind separately to AMPKα and AMPKγ, a homologue of Snf4p, and mediates formation of a ternary complex (Stapleton *et al.*, 1994; Woods *et al.*, 1996). Like its mammalian counterpart, Snf1p is activated by phosphorylation upon transferring cells from repressing conditions to derepressing conditions (Wilson *et al.*, 1996). It has been suggested that an upstream protein kinase most likely serves as an activator of Snf1p and a high AMP:ATP ratio may be a good candidate for the metabolic signal that triggers depression of glucose-repressed genes (Wilson *et al.*, 1996).

MIG1*, *SSN6* and *TUP1

MIG1 was identified as a multicopy inhibitor of *GAL* gene expression (Nehlin and Ronne, 1990). *MIG1* encodes a zinc-finger protein of 504 amino acids that represses transcription of *SUC2* and *GAL* genes (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991). Overexpression of *MIG1* reduces galactose fermentation and growth on raffinose, a substrate of invertase (*SUC*). Deletion of *MIG1* relieves glucose repression of *GAL4* and partially relieves repression of *GAL1*, *GAL2*, and *SUC2* expression. The promoters of *SUC2*, *GAL1* and *GAL4* each contain one or two Mig1p-binding sites (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991). Saturation mutagenesis of the Mig1p-binding site demonstrated that Mig1p binds to a GC-box but a flanking AT-rich region is also important for Mig1p-binding (Lundin *et al.*, 1994). It is possible that AT box acts to enhance binding of Mig1p to the GC box since Mig1p was found to bend DNA within the AT box. Deletion analysis of *MIG1* revealed that a C-terminal, proline-rich domain of 24 amino acids is necessary and sufficient for Mig1p repression (Ostling *et al.*, 1996). Two internal elements mediate the inhibition of Mig1p in the absence of glucose. LexA-Mip1 protein was found to be differentially phosphorylated in response to glucose (Treitel and Carlson, 1995).

The *ssn* mutations were isolated as suppressors of *snf1* mutations (Carlson *et al.*, 1984). *SSN1* gene was later shown to be the same as *MIG1* (Vallier and Carlson, 1994). The fact that *mig1Δ* partially suppresses the *SUC2* derepression defect of *snf1Δ* mutant suggests that Snf1p functions to prevent Mig1p from repressing its target genes in the absence of glucose (Vallier and Carlson, 1994).

Mutations in *SSN6* and *TUP1* cause similar pleiotropic defects in addition to glucose-resistant expression of glucose-repressible genes (Johnston and Carlson, 1992; Trumbly, 1992). Multiple copies of *SSN6* reduce derepressed expression of *SUC2* (Schultz and Carlson, 1987). *SSN6* encodes a protein of 966 amino acids (Schultz and Carlson, 1987; Trumbly, 1988). Ssn6p contains near its amino terminus a 10 copies of a 34-residue motif (tetratricopeptide repeat or TPR; Hirano *et al.*, 1990) that is functionally important (Schultz *et al.*, 1990). The polyglutamine and poly(glutamine-alanine) tracts of the protein appear to be dispensable and removal of a third of the protein at the carboxyl terminus does not cause a severe mutant phenotype (Schultz and Carlson, 1987). Because TPR motifs are present in functionally diverse proteins often associated in protein complexes, they are proposed to mediate protein-protein interaction (Hirano *et al.*, 1990; Goebl and Yanagida, 1991).

TUP1 gene codes for a 713-amino-acid protein (Williams and Trumbly, 1990). Tup1p contains six copies of a WD (also known as β-transducin) motif in the carboxyl-terminal portion of the protein. WD repeats are present in many proteins of diverse function including G_β subunit and are thought to mediate protein-protein interaction. Ssn6p and Tup1p are physically associate with each other (Williams *et al.*, 1991) and are required for repression of various unrelated genes (Keleher *et al.* 1992; Schultz and Carlson; 1987; Zitomer and Lowry, 1992; Elledge *et al.*, 1993). It has been proposed that Ssn6p/Tup1p is a general repressor that is recruited to target promoters by various pathway-specific DNA-binding proteins such as Mig1p (glucose repression), MATα2 protein (cell type regulation), and Rox1p (oxygen regulation) (Keleher *et al.*, 1992). Ssn6p and Tup1p appear to play key roles in organizing repressive chromatin structure (Cooper *et al.*, 1994).

Tup1p has been shown to perform the repression function of the Ssn6-Tup1 corepressor complex (Tzamarias and Struhl, 1994). Tup1p contains two independent repression domains: the 200 N-terminal residues and a region C-terminal to residue 288. The N-terminal 72 residues of Tup1p are necessary and sufficient for formation of Ssn6p-Tup1p complex. None of these domains includes the WD motif. A *TUP1* derivative lacking the WD motif can partially mediate repression of *SUC2* and *ANB1* (oxygen-regulated) transcription (Tzamarias and Struhl, 1994). However, the Tup1 WD repeats interact directly with $\underline{\alpha}2$ and are required for repression of cell type-specific genes (Komachi *et al.*, 1994).

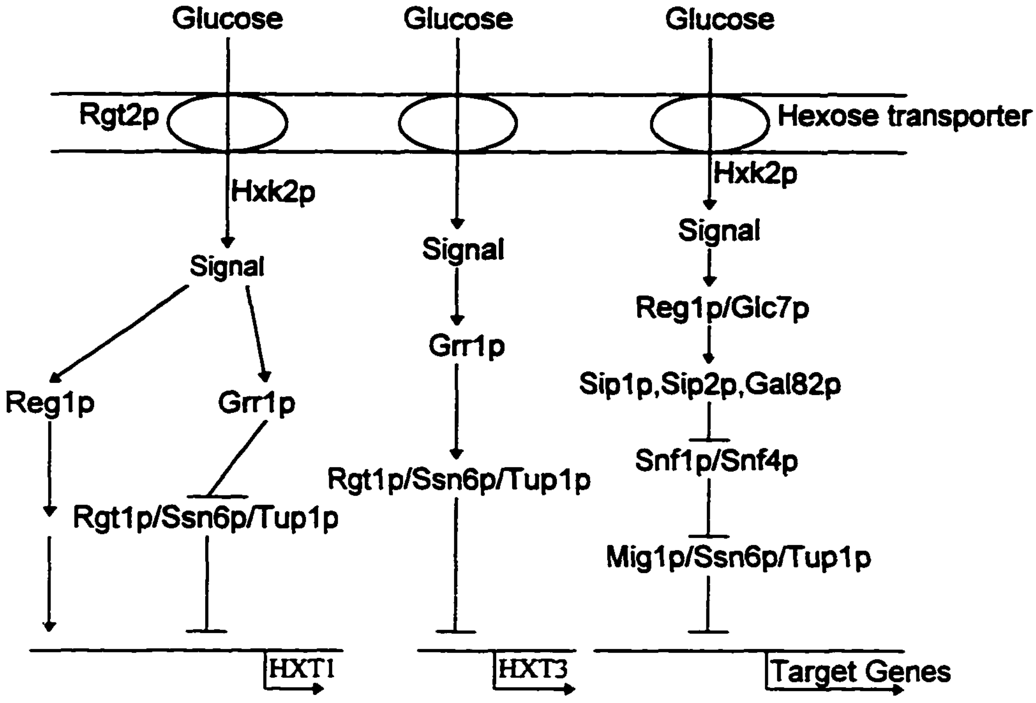
Tzamarias and Struhl (1995) showed that TPR1-3 of Ssn6p are necessary and sufficient to interact directly with residue 1-72 of Tup1p. The carboxyl-terminal 150 residues appear to be dispensable for Ssn6p function. The TPR repeats of Ssn6p are not functionally redundant despite the sequence similarities between them. TPR8, TPR9, and possibly TPR10, but not TPR4-7, are required for glucose repression. TPR4-7, but not TPR8-10, are required for oxygen repression. Repression by Mat$\underline{\alpha}$2 only requires TPR1-3 and repression of DNA-damage-regulated genes appears to require the entire TPR repeats and additional C-terminal sequences (Tzamarias and Struhl, 1995). Ssn6p plays an important role in recruiting Ssn6-Tup1 complex differentially to pathway-specific promoters. Tup1 derivative missing the N-terminal Ssn6p-binding domain partially represses the cell type-specific gene *MFA1* but not the others, consistent with the observation that WD motif of Tup1p interacts with MAT$\underline{\alpha}$2 (Tzamarias and Struhl, 1995; Komachi *et al.*, 1994).

Glucose repression by Mig1p depends on *SSN6* and *TUP1* (Treitel and Carlson, 1995). Interaction between Mig1p and Ssn6p was detected in the two-hybrid system. However, LexA-Mig1p activates transcription in a *ssn6Δ* and *tup1Δ* strain, suggesting that Ssn6p-Tup1p can also function to inhibit Mig1p transcriptional activity (Treitel and Carlson, 1995; Tzamarias and Struhl, 1995). Mutations in *SSN6* and *TUP1* cause a dramatic loss of glucose repression of *SUC2* while disruption of *MIG1* only partially relieves repression (Vallier and Carlson, 1994). Other *SSN* genes and *MIG1* may thus act synergistically in mediating glucose repression by *SSN6-TUP1* (Vallier and Carlson, 1994).

Genetic interactions of genes involved in glucose repression

Genetic analysis of suppression and epistasis among genes involved in glucose repression has provided a framework for this regulatory pathway. The results of those studies are diagrammed in Figure 2 and can be summarized as follows. *RGT1* and its negative regulator *GRR1* act at an early stage of glucose repression to control expression of hexose transporter (*HXT*) genes. Suppression of both the glucose repression-resistance and the high-affinity glucose transport defects in *grr1* mutants by *rgt1* mutations clearly points to a correlation between glucose transport and glucose repression (Erickson and Johnston, 1994). As a glucose-phosphorylating enzyme, Hxk2p is proposed to

A.



B.

Gene	Function
GRR1	Putative ubiquitin protein ligase
HXK2	Hexokinase
MIG1	Zinc-finger DNA-binding repressor
REG1	Regulatory subunit of protein phosphatase type-1
RGT1	Zinc-cluster DNA-binding repressor
RGT2	High-glucose sensor, glucose transporter homologue

Figure 2. A. Schematic model of the regulatory pathway for glucose repression of *SUC2* and *GAL* genes. B. Functions of genes that are involved in glucose regulatory pathways and examined in this study.

function at an early step in generating and/or transmitting glucose signal (Johnston and Carlson, 1992). Isolation of suppressors of *hxk2* mutations has not been successful (Erickson and Johnston, 1994), but *snf1* mutations are epistatic to *hxk2* and are thus downstream of *HXK2*. Mutations in *SNF1-6* (except *snf3*) are epistatic to *reg1* and *glc7* (Entian and Zimmermann, 1982; Neigeborn and Carlson, 1984, 1987). Glucose regulation of Snf1p-Snf4p interaction appears to involve *HXK2* and *GLC7/REG1*, supporting that these three genes act upstream of *SNF1* (Jiang and Carlson, 1996). *HXK2* is also required for full induction of *HXT2-4* genes by low levels of glucose (Ozcan and Johnston, 1995). Both *HXK2* and *REG1* appear to be involved in high-glucose induction of *HXT1* by a mechanism that is independent of *RGT1* (Ozcan and Johnston, 1995; Ozcan *et al*., 1996). High-glucose induced expression of *HXT3* does not require *HXK2* and *REG1*, and thus may be controlled differently from that of *HXT1* (Ozcan and Johnston, 1995).

GAL82-1 and *GAL83-2000* mutations specifically affect glucose repression of *GAL* genes (Erickson and Johnston, 1993). A low copy clone of *REG1* cross-complements *GAL82-1* and *GAL83-2000*. The *set1* mutation only suppresses *GAL82-1* (Erickson and Johnston, 1994). The *reg1*, *GAL82-1* and *GAL83-2000* mutations fail to complement each other but all three mutations can be suppressed by *srg1* mutation, suggesting that three genes are functionally related (Erickson and Johnston, 1993, 1994). Moreover, *snf1* mutations are epistatic to *GAL82-1* and *GAL83-2000* while *SIP1* and *SIP2* on a high-copy plasmid cross-complement *GAL82-1* and *GAL83-2000* mutations. These results are consistent with the model in which the products of these genes act reduntantly to regulate Snf1p activity (Erickson and Johnston, 1993).

The *ssn* mutations are epistatic to *snf1* and *snf4* (Carlson *et al.*, 1984; Neigeborn and Carlson, 1987). The *tup1* and *ssn6* mutations cause similar phenotypes and are functionally related (Neigeborn and Carlson, 1987). Snf1p appears to inhibit (directly or indirectly) the activity of the Mig1p(Ssn1p)-Ssn6p-Tup1p complex. *SSN2-5*, *SSN7*, and *SSN8* genes may also mediate repression by Ssn6-Tup1 co-repressor (Vallier and Carlson, 1994).

Genes that are repressed by glucose

The expression of genes required for galactose metabolism is induced by galactose and repressed by glucose (reviewed in Lohr *et al.*, 1995). These genes includes *GAL1*, *GAL7*, and *GAL10* which encodes enzymes of the Leloir pathway, as well as *GAL2* encoding galactose permease and *GAL3*. Galactose induction is mediated by *GAL4*, *GAL80* and *GAL3*. *GAL4* encodes a zinc cluster transcription factor which binds to the promoters of *GAL* structural genes constitutively and activates transcription in response to galactose. Gal80p interacts with the activation domain located in the C-terminus of Gal4p and inhibits its activity in the absence of galactose. *GAL3* encodes a protein homologous to *GAL1*-encoded galactokinase (Suzuki-Fujimoto *et al.*, 1996). Galactose causes a strong interaction between Gal3p and Gal80p in the presence of ATP probably by binding to Gal3p (Zenke *et al.*, 1996; Suzuki-Fujimoto *et al.*, 1996; Blank *et al.*, 1997; Yano and Fukasawa, 1997). Binding of Gal3p to Gal80p in turn relieves inhibitory effect of Gal80p on Gal4p.

Three mechanisms mediate glucose repression of *GAL* genes: (i) reduction of cellular Gal4p levels; (ii) repression via *cis*-acting elements located in the *GAL* promoters; (iii) inhibition of induction by galactose (Trumbly, 1992; Johnston and Carlson, 1992; Johnston *et al.*, 1994). Transcription of *GAL4* is repressed by Mig1p in the presence of glucose (Nehlin *et al*, 1991; Griggs and Johnston, 1991) resulting in an absence of Gal4p binding to UAS_{GAL} (Huibregtse *et al.*, 1993). Glucose causes transcriptional repression of *GAL* structural genes through URS_{GAL} elements (Flick and Johnston, 1990, 1992). One type of the URS_{GAL} elements is the Mig1p-binding sites located in *GAL1* and *GAL2* promoters (Nehlin *et al.*, 1991). The *GAL80*-dependent mechanism (Nehlin *et al*, 1991; Lamphier and Ptashne, 1992) reflects the inhibition of galactose induction by glucose. The expression of *GAL3* required for galactose induction is repressed by glucose (Bajwa *et al.*, 1988). *GAL2* encoding galactose permease is subject to both glucose repression (Nehlin *et al*, 1989, 1991; Huibregtse *et al.*, 1993) and glucose-induced proteolysis (Matern and Holzer, 1977; DeJuan and Lagunas, 1986). Glucose might interfere with galactose induction by affecting the interaction between Gal3p and Gal80p (Tano and Fukasawa, 1996). However, overexpression of *GAL4* (which confers Gal4p constitutive activity) leads to Gal4p binding to UAS_G but not transcriptional activation of *GAL2* in the presence of glucose (Huibregtse *et al.*, 1993). The central region of Gal4p has been shown to mediate a direct inhibition of Gal4p activity by glucose (Stone and Sadowski, 1993). *ADA3* encodes a component of Ada2-Ada3-Gcn5 transcription coactivator complex (Horiuchi *et al.*, 1995) and appears to be involved in inhibition of Gal4p function by glucose (Brandl *et al.*, 1993).

The enzyme invertase, which hydrolyses extracellular sucrose and raffinose into glucose and fructose, is encoded by the *SUC* gene family (Carlson and Botstein, 1983). The most commonly found and extensively characterized *SUC* gene is *SUC2*. The transcription of *SUC2* is glucose repressed (Carlson and Bostein, 1982, 1983). Two general types of mutants affecting *SUC2* expression have been isolated: derepression mutants such as *snf* mutations and repression mutants such as *hxk2*, *reg1*, *grr1*, and *ssn* mutations (reviewed in Trumbly, 1992; Johnston and Carlson, 1992). One surprising discovery from the study of glucose repression of *SUC2* was that *SUC* regulation is reversed in *grr1* mutants (Vallier and Carlson, 1991; Flick and Johnston, 1991). Mutations in *GRR1* convert *SUC2* from a glucose-repressible gene to a glucose-induced gene. However, normal regulation of *SUC2* is restored in *grr1 rgt1* double mutants (Vallier *et al.*, 1994). It was subsequently found that transcription of *SUC2* is induced to maximal levels by low concentrations of glucose and this is dependent upon *SNF3* (Ozcan *et al.*, 1997). Altered responses to glucose are apparently associated with the high-affinity glucose transport defect in *grr1* mutants. Relief of glucose repression in *grr1* mutants reveals the glucose-induced *SUC2* expression at high glucose levels (Ozcan *et al.*, 1997).

Lutfiyya and Johnston (1996) recently identified a gene, *MIG2*, which appears to act on *SUC2* but not other glucose-repressed genes. Mig2p contains two zinc fingers that are highly similar to those of Mig1p and binds to the Mig1p-binding sites in the *SUC2* promoter (Lutfiyya and Johnston, 1996). Glucose repression by Mig2p also requires Ssn6p and Tup1p. Since *SUC2* expression is still regulated by glucose in *snf1Δ mig1Δ* and *snf4Δ mig1Δ* mutants, the glucose signal can be transmitted independent of Snf1p kinase to possibly other Ssn proteins or Mig2p that act on *SUC2* promoter (Vallier and Carlson, 1994). In addition to transcriptional control, the stability of *SUC2* mRNA has been shown to be affected by glucose and this regulatory process involves *REG1* and any one of hexokinases (Cereghino and Scheffler, 1996).

ADH2 encodes alcohol dehydrogenase isozyme II responsible for utilization of ethanol. *ADH2* is severely repressed during growth on glucose (Denis *et al.*, 1981). Expression of *ADH2* in ethanol media is mediated by *ADR1* which encodes a DNA-binding transcription activator (Denis and Young, 1983; Eisen *et al.*, 1988) and requires the general transcription factor *CCR4* (Denis, 1984; Malvar *et al.*, 1992). Adr1p function is inhibited by glucose (Wills, 1990). Transcription of *ADR1* is repressed by glucose in some strains but not in others (Blumberg *et al.*, 1988). A reduction of protein synthesis of Adr1p also is observed in some strains (Vallari *et al.*, 1992). Among many genes affecting glucose repression of *SUC2* and *GAL*, only *REG1* is involved in glucose regulation of *ADH2* apparently at multiple levels (Domek *et al.*, 1993). Although *SNF1* is required for activation of *ADH2* (Denis, 1987), its targets appear to be different from those for *SUC2* expression since *SSN6* and *TUP1* do not seem to affect *ADH2* expression (Dombek *et al.*, 1993). Ccr4p contains leucine-rich repeats and is a component of a transcriptional regulatory complex including Pop2p (Draper *et al.*, 1994, 1995; Sakai *et al.*, 1992). Transcriptional activation by Ccr4p also is regulated by glucose (Draper *et al.*, 1994).

The development of mitochondria and an active respiratory system is induced by oxygen and repressed by glucose (reviewed in Fosberg and Guarente, 1989; Trumbly, 1992). Transcriptional activation of *CYC1* which encodes an isoform of cytochrome c is mediated by both *HAP1* in a heme-dependent manner and the Hap2-Hap3-Hap4 protein complex. Expression of *HAP2* and *HAP4* is repressed by glucose (Pinkham and Guarente, 1985). Glucose repression of *CYC1* is also controlled by *SNF1*, *SSN6*, and *REG1* (Wright and Poyton, 1990; Borralho *et al.*, 1989).

Glucose repression of the gluconeogenic genes seems to involve multiple mechanism (Ronne, 1995). However, *ssn6* and *tup1* mutations have little effect on glucose repression of those genes. *FBP1* and *PCK1*, encoding fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, respectively, are controlled by transcriptional repression in response to even extremely low concentrations of glucose (Mercado *et al.*, 1991, 1994; Yin *et al.*, 1994). This repression can be mediated by any one of the three glucose-phosphorylating enzymes but is missing in a triple *hxk1 hxk2 glk1* mutant (Yin *et al.*, 1994). Mig1p-dependent pathway, although operative, appear to be not essential for glucose repression of *FBP1* and *PCK1*. Glucose also affects the turn-over of *PCK1* mRNA (Mercado *et al.*, 1994). In addition, fructose 1,6-bisphosphatase is subject to glucose-induced inactivation (reviewed in Johnston and Carlson, 1992).

Part II. Maltose Fermentation in *Sacchromyces cerevisiae*

Maltose fermentation in *Saccharomyces cerevisiae* requires two enzyme functions, maltose permease which transport maltose into the cells and maltase which hydrolyzes maltose to produce glucose. Expression of maltose permease and maltase genes is induced by maltose and repressed by glucose (reviewed in Needleman, 1991). Maltose induction requires a positive regulatory protein, the *MAL*-activator (Chang *et al.*, 1988). *S. cerevisiae* strains capable of fermenting maltose possess at least one of the five unlinked *MAL* loci, *MAL1* through *MAL4*, and *MAL6* (Michels and Needleman, 1983; Needleman and Michels, 1983; Michels and Needleman, 1984; Naumov *et al.*, 1994). Each of the *MAL* loci is located near the telomere of different chromosomes, *MAL1* (chromosome VII), *MAL2* (chromosome *III*), *MAL3* (chromosome *II*), *MAL4* (chromosome *XI*), and *MAL6* (chromosome *VIII*) (reviewed in Barnett, 1981). All five *MAL* loci are highly homologous not only at sequence level but also functionally (Charron *et al.*, 1989). The repeated copies of *MAL* family of loci may have resulted from telomeric translocation (Charron *et al.*, 1989; Michels *et al.*, 1992; Naumov *et al.*, 1994). Each *MAL* locus is a complex of three genes, Gene 1 encoding maltose permease, Gene 2 encoding maltase, and Gene 3 encoding the *MAL*-activator. The orientation of three genes was determined as: Centromere ... Gene 3 – Gene 1 – Gene 2 ... Telemere (Charron *et al.*, 1989). Gene 1 and Gene 2 share a bidirectional promoter (the intergenic region) (Levine *et al.*, 1992; Yao *et al.*,

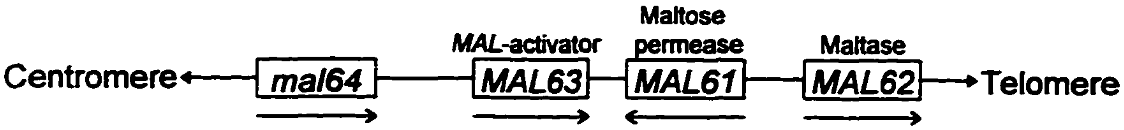


Figure 3. The *MAL6* locus.

1994). Nomenclature distinguishing each of the three genes at various *MAL* loci utilizes a two-digit numbering system which defines both classical chromosome designation and position-function of a particular *MAL* gene. For example, *MAL62* encodes maltase at *MAL6* (Figure 3).

Naturally occurring partially functional *MAL* alleles have been identified at *MAL1* and *MAL3* (Naumov, 1969, 1971, 1972, 1976; Oshima, 1967; Michels and Needleman, 1983). There are three partially functional alleles of *MAL1*: *MAL1p* which only expresses the *MAL*-activator, *MAL1g* which expresses both maltose permease and maltase, and *mal1*^{0} which only expresses maltase (Charron and Michels, 1988). However, the functional maltose permease encoded by *MAL1g* surprisingly exhibits little sequence similarity at the DNA level to *MAL11*. All naturally existing *S. cerevisiae* strains that have been analyzed genetically appear to contain an allele of *MAL1*, suggesting that *MAL1* may be the progenitor of the *MAL* family (Charron and Michels, 1988; Naumov *et al.*, 1994). The *MAL3g* alleles contains *MAL31* and *MAL32* but lacks a functional *MAL33* gene (Michels *et al.*, 1992).

A non-functional homologue of *MAL63*, *mal64*, has been identified at *MAL6* locus (Dubin *et al.*, 1986). The *mal64* gene is located upstream and centromere proximal to *MAL63*, and can be activated by a mutation to encode a constitutive *MAL*-activator (Dubin *et al.*, 1988). A *mal64* homologue sequence also is present at *MAL3* but whether or not it is functional has not been determined (Michels *et al.*, 1992). An inducible *MAL43* allele is not available but the *MAL*-activator gene cloned from a constitutive *MAL4* strain encodes a dominant constitutive activator, Mal43-Cp (Charron and Michels, 1987).

MAL61 and *MAL11* encode a high-affinity maltose/proton symporter whose expression is induced by maltose (Chang *et al.*, 1989; Cheng and Michels, 1991). Transcription of *MAL61* and *MAL11* produces two transcripts, a maltose-induced 2.0-kb transcript and a partially constitutive 2.4-kb transcript (Needleman *et al.*, 1984; Charron *et al.*, 1986). Mal61p is a 614-residue protein and is a member of the twelve transmembrane domain family of sugar transporters (Cheng and Michels, 1989). Both the 100 N-terminal residues and the 67 C-terminal residues are predicted to be located on the cytoplasmic face of the plasma membrane. Two blocks of six transmembrane domains are thus separated by an intracellular region of approximately 71 residues. Mal61p is homologous to other sugar transporters including the *S. cerevisiae* galactose permease, Gal2p, and the glucose transporter homologue Snf3p. Disruption of *MAL11* in a strain containing only a *MAL1* locus abolishes maltose-induced expression of *MAL12* while disruption of *MAL12* has no effect on the expression of *MAL11* (Charron *et al.*, 1986). The same results were obtained in a *MAL6* strain (Dubin, 1987). Thus, a functional maltose permease gene is required for *MAL*-activator-mediated maltose induction.

The gene encoding a functional maltose permease at the *MAL1g* allele is referred as *AGT1* (α-glucoside transporter) (Han *et al.*, 1995). *AGT1* encodes a 617 amino acid protein which is 57% identical to Mal61p and belongs to the twelve-transmembrane domain superfamily of sugar transporters. Agt1p is a high-affinity maltose transporter. However, the substrate specificity of Agt1p is broader than that of Mal61p which only transport maltose and turanose. Besides maltose and turanose, Agt1p is capable of transporting other α-glucosides including isomaltose, α-methylglucoside, maltotriose, palatinose, trehalose and melezitose. The expression of *AGT1* is induced by maltose and is dependent on the *MAL*-activator. The 785 basepair *AGT1-MAL12* intergenic region is 90 bp shorter than the *MAL61-MAL62* region and the 315 basepair immediately upstream of *AGT1* exhibit no significant sequence homology to the corresponding region of *MAL61*. The rest of the sequences in these two intergenic regions are almost 100% identical including the $\mathrm{UAS}_{\mathrm{MAL}}$ crucial for transcriptional activation by the *MAL*-activator (Levine *et al.*, 1992). However, a constitutive *MAL*-activator gene is required in order for strains carrying *AGT1*, *MAL12*, and *MAL13* to ferment trehalose, melezitose, and to a lesser extent, α-methylglucoside, but not the other sugars mentioned above. This probably reflects the difference in the abilities of these various α-glucosides to induce the *MAL*-activator (Han *et al.*, 1995).

Maltose-induced transcription of *MAL12* and *MAL62* each produces a single 1.9-Kb transcript (Needleman *et al.*, 1984; Charron *et al.*, 1986). *MAL62*-encoded maltase is a 584-residue cytoplasmic protein (Hong and Marmur, 1986). Mal62p is able to hydrolyze a broad spectrum of α-glucosides including maltose, turanose, and sucrose (Needleman *et al.*, 1978). Maltase shows sequence homology to amylase and transglucanosylase (Svensson, 1988).

MAL63 synthesizes two maltose-induced transcripts, 1.6 kb and 2.0 kb (Needleman *et al.*, 1984) while transcription of *MAL13* only produces a 1.6-kb transcript (Charron *et al.*, 1986). Mal63p is a 470-residue transcriptional activator of the *MAL* structural genes (Chang *et al.*, 1988; Kim and Michels, 1988). A Cys_6 zinc cluster DNA-binding motif is present at the amino terminus of Mal63p which is highly homologous to those of a family of yeast transcriptional factors (Schjerling and Holmberg, 1996). A fragment of the first 111 residues of Mal63p containing the zinc cluster and adjacent region and expressed in *E. coli* is able to bind to a 40 bp DNA fragment from UAS_{MAL} in a gel mobility shift assay (Kim, 1992). Deletion analysis of the shared, bidirectional *MAL61-MAL62* promoter reveals a 68 basepair upstream activation sequence, the UAS_{MAL}, between basepair -515 and -582 upstream of the *MAL61* start codon necessary for the maltose-induced transcription of *MAL61* and *MAL62* (Levine *et al.*, 1992). Mal63p appears to bind to three sites in the *MAL61-62* promoter (Sirenko *et al.*, 1995; Wang and Needleman, 1997). Site 1 and site 2 each have the consensus sequence $CGC/GN_9CGC/G$ and site 3 is a half site. However, deletion of a region containing site 2 does not appear to affect maltose-induced transcription of the *MAL* structural genes (Levine *et al.*, 1992). Mal63p overexpressed and purified using DNA-affinity chromatography from yeast cells grown in uninduced conditions appears to form a dimer in solution (Sirenko *et al.*, 1995).

Two dominant constitutive *MAL*-activator mutations were mapped to *mal64*, a nonfunctional homologue of *MAL63* (Dubin *et al.*, 1986, 1988). Maltose-induced transcription of *mal64* depends on *MAL63* while the two constitutive mutants are transcribed constitutively (Dubin *et al.*, 1988). The wild-type *mal64* gene encodes a protein of predicted length of 470 residues which is 85% identical to Mal63p (Gibson *et al.*, 1997). Two constitutive mutants, *MAL64-R10* and *MAL64-C2*, contains a nonsense mutation at codons 282 and 307, respectively. Their constitutive phenotype results from the truncation of the C-terminal portion of Mal64p. Studies using hybrid genes of *MAL63* and *mal64* show that the non-functional phenotype of *mal64* is due to sequence alterations in the C-terminal half of the mal64 protein.

Both the dominant constitutive *MAL43-C* and the wild-type *MAL23* genes encode a 470-residue protein (Gibson *et al.*, 1997). Mal23p has 20 altered residues compared to Mal63p. Mal43-Cp contains 24 and 31 variant residues as compared to Mal23p and Mal63p, respectively. A *MAL63/43-C* hybrid gene in which the C-terminal 255 residues of *MAL43* are fused to the N-terminal 215 residues of *MAL63* shows the same constitutive phenotype as *MAL43-C*, suggesting that the C-terminal portion of the *MAL*-activator contains a negative regulatory domain. The *MAL63* constitutive mutants also contain mutations located in the C-terminal region of the protein (Wang and Needleman, 1996). The constitutive *MAL23-C* varies significantly from wild-type *MAL23* in a region between residues 322-378 of the protein (Gibson *et al.*, 1997). A *LexA-MAL63*$_{(1-}$

283) fusion expressed from an *ADH1* promoter also functions as a constitutive *MAL*-activator (Gibson, 1995). Thus, the N-terminal region of the *MAL*-activator appears to possess the DNA-binding and trans-activation functions, and the C-terminal region contains a maltose-responsive regulatory domain.

It is noteworthy that some mutations shared by the constitutive *MAL43-C* and *MAL63-C* are also found in the wild-type inducible *MAL23*. Other sequence variations between Mal23p and Mal63p may be responsible for the suppression of those mutations in *MAL23* that cause constitutivity of Mal63p (Danzi, 1997). The putative *MAL13* and *MAL33* genes have been cloned and sequenced from a strain which does not ferment maltose (Feuermann *et al.*, 1995; Volckaert *et al.*, 1997). Whether these two cloned alleles are functional is thus in question.

Expression of the *MAL* genes is repressed by glucose (Needleman *et al.*, 1984). *SNF1*, a central component of glucose repression regulatory pathway, is required for utilization of maltose (Carlson *et al.*, 1981). Deletion analysis of the *MAL61-62* intergenic region identified a region next to the UAS_{MAL} which partially mediates glucose repression of *MAL62* (Levine *et al.*, 1992). This region exhibits sequence homology to the URS_G element of the *GAL1* promoter which has been shown to bind Mig1p repressor (Flick and Johnston, 1992; Nehlin *et al*, 1991). A high-copy plasmid carrying *MIG1* gene also inhibits growth on maltose (Nehlin and Ronne, 1990) suggesting that Mig1p may function as a repressor of *MAL* gene expression in response to glucose. The constitutive *MAL43-C* and *MAL23-C* mutants show glucose insensitivity while the constitutive *MAL64-R10* and

MAL64-C2 are partially sensitive to glucose (Charron and Michels, 1987; Gibson *et al.*, 1997). This glucose insensitivity of the constitutive *MAL*-activator genes may reflect a relationship between maltose induction and glucose repression.

In addition to glucose repression, maltose permease is subject to glucose-induced inactivation (reviewed in Johnston and Carlson, 1992). Glucose inactivation of maltose permease assayed in nitrogen-starvation media exhibits a biphasic process, consisting of a rapid loss of transport activity (inhibition) followed by a slow decrease in the amount of permease protein (proteolysis) (Medintz *et al.*, 1996). The proteolysis of Mal61p depends on endocytosis and vacuolar proteolysis but is independent of proteosome.

Materials and Methods

Strains and growth media. The *S. cerevisiae* strains used throughout this study are 332-5A (*MAL61 MAL62 MAL63 mal64 mal11 MAL12 mal13 ura3-52 leu2-3,112 trp1 his)* (Dubin *et al.*,1988), YPH500 (*MATα AGT1 MAL12 mal13 MAL31 MAL32 mal33Δ ura3-52 his3Δ200 leu2-Δ1 trp1-Δ63 lys2-801 ade2-101)*, KT1357 (*MAT***a** *AGT1 MAL12 mal13Δ MAL31 MAL32 mal33Δ ura3-52 leu2 his3 trp1*) (Frederic and Tatchell, 1996), CMY1001 (*MAT***a** *MAL61/HA MAL12 MAL13 leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his2-Δ200)* (Medintz *et al.*, 1996). Strain R10 is isogenic to 332-5A except that it contains the constitutive *MAL*-activator allele *MAL64-R10* plus a deletion of *MAL63* constructed by removal of most of the ORF and insertion of the *URA3* gene (Dubin *et al.*, 1988). Strains CMY1006, CMY1015 are isogenic to CMY1001 except that they carry a *hxk2::URA3* and *hxk2::URA3 hxk1::TRP1* deletion, respectively (Medintz *et al.*, 1996; Jiang, 1997). Strains CMY1005 and CMY1009 are isogenic to CMY1001 except for a *grr1::LEU2* and a *rgt2::*kan^R disruption respectively (Jiang *et al.*, 1997). Strain CMY1010 is a *rgt1::URA3* derivative of CMY1005 (Jiang *et al.*, 1997). Strain R10u and CMY1006u are the *ura3* derivatives of R10 and CMY1006, respectively, isolated on 5-fluoro-orotic acid-containing medium (Boeke *et al.*, 1984). Strain DF186 is isogenic to KT1357 except that *REG1* was disrupted by a *URA3* gene (Frederic and Tatchell, 1996). Yeast strains were grown at 30°C in either rich media (1% yeast extract, 2% peptone) or minimal medium (0.67% yeast nitrogen base with appropriate amino acids and nitrogen base supplements) plus various carbon sources as specified.

Normally used *E. coli* strains were RR1 and DH5α grown in LB medium or LB medium plus ampicillin at 37°C. *E. coli* CJ236 and MV1190 were used in site-directed mutagenesis (BioRad). MV1190 was grown in YT medium or M9 medium as required and CJ236 in LB medium plus chloramphenicol at 37°C.

Oligonucleotides. A list of oligonucleotides utilized throughout this work is presented below.

Oligonucleotide	Sequence (5'--3')
Z1	GGGGGATCCTGGTTCTGTTTCTGGATG
Z2	GGGGGATCCGGATAATCCCTTCAT
Z3	GCCTCTGTGGAAACTTC
Z4	CCTTGGCTATAGTAAG
Z5	CCTAGCGTACCTAACG
Z6	CTACAACAACGTCTTAAAAATTATGTGGAG
Z7	GGAAATTTTCGCGGAACAATTAAGAATTGGC
Z8	CGTTAGGTACGCTAGG
Z9	GGGGAATTCAGGGGTTCCGCGCACAT
Z10	GGGGAATTCCACCGTCATCACCGA
L1	GGAGATCTCATAAACGACATTACTAT
L2	GGGGTACCTGATGCGGTATTTTCTCC

Plasmids. Plasmids pHR81 and pMIG1 have been described previously (Nehlin and Ronne, 1990). The *ARSH4/CEN6* cassette was obtained by PCR using oligonucleotides Z9 and Z10 from plasmid pRS315 (Stratagene) and then inserted into the *Eco*RI site of plasmid YIp355 (Myers *et al.*, 1986), resulting in plasmid YCp355. The orientation of the *ARSH4ICEN6* in YCp355 was confirmed by PCR using oligonucleotides Z9 and Z8 after insertion of the *MAL61-62* intergenic region. The ability of YCp355 to function as a stable, episomal plasmid was confirmed by yeast transformation followed by plasmid segregation. Plasmid pHR81/MAL63 was constructed by inserting a 1.7kb *Bam*HI fragment containing *MAL63* into pHR81.

Plasmid pUN30/MAL23 was created by inserting the *Bgl*II-*Sal*I fragment from the *MAL23* locus into pUN30 digested with *Bam*HI and *Sal*I. A fragment containing the *TRP1* gene was obtained from plasmid pRS304 by PCR using oligonucleotides L1 and L2 which carries *Bgl*II and *Kpn*I site, respectively. This *TRP1* fragment was inserted into pSH2-1/MAL63$_{(2\text{-}216)}$ digested with *Bgl*II and *Kpn*I, creating plasmid pLexA/MAL63$_{(2\text{-}216)}$. A *Eco*RI-*Bam*HI fragment containing codons 216-470 of *MAL63* was inserted into pLexA/MAL63$_{(2\text{-}216)}$, resulting in plasmid pLexA/MAL63 carrying the *TRP1* gene for selection of yeast transformants. Plasmid pSH2-1/MAL63 is a *HIS3* vector with *MAL63*$_{(2\text{-}470)}$ fused to *LexA* (Gibson, 1995). Plasmid pBM3270 (obtained from M. Johnston) is a *CEN* plasmid carrying *URA3* and the dominant *RGT2-1* allele.

Mutagenesis and construction of *LacZ* fusions. A 0.9-kb *Bam*HI fragment containing the entire intergenic region of *MAL61-62* (Levine *et al.*, 1992), including a few codons at the beginning of both structural genes, was created by PCR using oligonucleotides Z1, Z2, and plasmid pY6 as a template (Needleman *et al.*, 1984). The *Bam*HI sites of these two oligonucleotides were designed so that each end of the resulting fragment could be fused, in-frame, to the *LacZ* gene in YCp355. This fragment was then subcloned into the *Bam*HI site of M13mp19 and the entire sequence was confirmed by sequencing using oligonucleotides Z3, Z4, and Z5. Site-directed mutagenesis was carried out using the Muta-Gene Kit (BioRad) according to the manufacturer's instructions. Internal deletions of the two Mig1p-binding sites were created with oligonucleotides Z6 and Z7 which exactly looped out the 6-bp GC box (Lundin *et al.*, 1994) of the corresponding Mig1p-binding site and inserted no additional nucleotides. Sequences were confirmed using a DNA Sequencing Kit (USB) according to the manufacturer's instructions. The *Bam*HI fragments containing the entire intergenic region were inserted in both orientations into the *Bam*HI site of the reporter plasmid YCp355 to create in-frame fusions to the *LacZ* gene.

Gene disruption and Southern analysis. *MIG1* and *SKO1* were disrupted in *S. cerevisiae* strains using plasmids pJN22 and pHR97, respectively, as described (Nehlin and Ronne, 1990; Nehlin *et al.*, 1992). pJN22 and pHR97 were digested with *Sac*I and *Xho*I, respectively, prior to yeast transformation. Disruptions of *MIG1* and *SKO1* were confirmed by Southern analysis of the genomic DNA digested with *Kpn*I and *Xho*I, respectively. *HXK2* was disrupted in strain YPH500 using plasmid pRB528 (obtained from David Botstein) which was digested with *Eco*RI prior to transformation (The *HXK2* disruptions in YPH500 were constructed by Bin Zhang in our laboratory). Plasmid pRB528 carries a *hxk2::URA3* deletional disruption. Genomic DNA of the putative *hxk2*Δ strains was digested with *Eco*RI for Southern analysis.

Northern analysis. Yeast cells were harvested in mid-log phase. Total RNA was isolated as described in Ausubel et al.(1994) and separated on 1.2% agarose-formaldehyde gels. After transfer to Hybond-N nylon membranes (Amershan, IL), the RNA was UV-crossed and hybridized as described previously (Nehlin and Ronne, 1990). Probes for the *MAL63*, *MAL61*, and *MAL62/12* genes were derived from plasmids pHR81/MAL63, pE2, and pD5, respectively (Dubin *et al.*, 1988). The amount of the hybridizing RNA was quantified in a Molecular Dynamics 400s PhosphorImager as described previously (Nehlin *et al.*, 1992).

Western analysis. Cells were harvested in mid-log phase. Whole-cell protein extracts were prepared as described (Ausubel *et al.*, 1994). Equal amounts of protein samples were separated in SDS-polyacrylamide gel and transferred to Hybond-ECL nitrocellulose membrane (Amersham) as described (Ausubel *et al.*

1994). Membranes were probed with anti-LexA antibody (obtained from Roger Brent) followed by horseradish peroxidase-linked donkey anti-rabbit antibodies (Amersham). Protein bands were visualized using the enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham) on ECL-Hyperfilm. The protein blots were quantified by scanning with a Beckman DU640 spectrophotometer.

Gel-shift assay. Double-stranded oligonucleotides were prepared as previously described (Nehlin *et al.*, 1992). The oligonucleotides were end-labeled with ^{32}P-dCTP using Klenow polymerase and purified on Biospin columns (BioRad) prior to use. *In vitro*-translated Mig1 and Sko1 proteins were prepared as described (Nehlin and Ronne, 1990). The Mig1p was incubated with ^{32}P-labelled oligonucleotides for 1 h at 20°C in Mig1p-binding buffer (Nehlin and Ronne, 1990). The reactions contained 1µl of Mig1p lysate, 10-30 pg (3000 cpm) of labeled DNA, and 0.5 mg of poly(dI:dC) carrier DNA (Pharmacia LKB) in a total volume of 10 µl. Protein-DNA complexes were separated on 6% polyacrylamide gels, which were then dried and autoradiographed (Nehlin *et al.*, 1992). Agarose-gel shift assays with end-labeled restriction fragments were performed as described previously (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991).

Enzyme assays. Yeast cells were grown in rich media or minimal media with 3% glycerol and 2% lactate (v/v) plus 2% of the specified sugar(s) (w/v) and harvested in mid-log phase. Maltase activities were determined as described (Dubin *et al.*, 1988). Maltase activity is expressed as nmoles of *p*-nitrophenol-α-glucopyranoside (PNPG) hydrolyzed per min per mg of protein. The assays of β-galactosidase activity were carried out in permeabilized cells and the activity was normalized to the cell density (Guarente, 1983; Ausubel *et al.*, 1994). Alternatively, β-galactosidase activities were measured in total cell extracts and the activity was then normalized to protein concentration for calculating specific activity (Rose *et al.*, 1990). Assay values are the average of results obtained from two to four independent transformants. Maltose transport activities were measured by the uptake of [^{14}C]maltose as described (Cheng and Michels, 1991). Inactivation protocol for assays of glucose sensitivity of maltose permease has been described in detail in Medintz *et al.*, 1996.

RESULTS

Part I. *MIG1*-Dependent Glucose Repression of *MAL* Gene Expression

Effect of the *MIG1* gene product on maltase gene expression. The *MAL6* strain 332-5A was transformed with plasmid pMIG1, which carries the *MIG1* gene in the *LEU2-d* defective plasmid pHR81. In leucine-deficient media, this plasmid amplifies to approximately 200-400 copies/cell leading to abundant expression of the *MIG1* gene (Nehlin and Ronne, 1990). Strain 332-5A, carrying pMIG1 and grown in the absence of leucine, exhibits dramatically reduced levels of maltase in both induced (2% maltose, 3% glycerol and 2% lactate) and uninduced (3% glycerol and 2% lactate) growth conditions (Table 1). These results are comparable to those obtained by Nehlin and Ronne (1990) who found that this amplified plasmid severely inhibited growth on galactose and raffinose but not glucose, and strongly suggest that *MIG1* is involved in the regulation of maltose fermentation as well as utilization of galactose and sucrose.

Deletion of *MIG1* in strain 332-5A led to slightly increased maltase levels in induced conditions but no effect was seen in cells grown in uninduced conditions (Table 2). The *mig1* disruption relieved glucose-repression and increased maltase expression in repressed (2% glucose, 3% glycerol and 2% lactate and induced/repressed (2% maltose, 2% glucose, 3% glycerol and 2% lactate) cells 40-60 fold. These results confirm the involvement of *MIG1* in the

TABLE 1

Effect of *MIG1* overexpression on maltase expression

	Maltase activity			
Plasmid	–	Mal	Glu	Mal+Glu
pHR81	7.8	441	0.8	2.0
pMIG1	1.4	26	1.7	4.2

Strain 332-5A was transformed with plasmid pMIG1 or the vector control plasmid, pHR81, and maltase levels determined as described in Dubin *et al.*, 1988. Cells were grown in minimal media lacking leucine plus 3% glycerol and 2% lactate (v/v) and 2% (w/v) of the indicated sugar(s).

TABLE 2

Effect of a *MIG1* deletion on maltase expression

Relevant genotype	Maltase activity –	 Mal	 Glu	 Mal+Glu
MIG1	4.5	350	0.5	0.8
mig1Δ	4.7	570	31	32

A deletion/disruption of the *MIG1* gene was constructed in strain 332-5A as described in Materials and Methods. Strains were grown in rich media plus 3% glycerol and 2% lactate and 2% of the indicated sugar. Maltase was assayed as described in Table 1.

glucose repression of maltase expression. Moreover, they demonstrate a six-fold induction of maltase activity by glucose in the *mig1Δ* strain compared to the level of expression in uninduced cells. A transient glucose-induced expression of *SUC2* is seen in a *mig1Δ* strain (Nehlin et al., 1992).

Effect of *MIG1* on the expression of the *MAL61*, *MAL62*, and *MAL63* mRNAs. The expression of the *MAL* genes was further investigated by Northern-blot analysis of total RNA isolated from strain 332-5A and an isogenic *mig1Δ* strain. The probes utilized were specific for the *MAL61*, *MAL62/12* and *MAL63* mRNAs expressed by this strain. Yeast cells were grown in glycerol/lactate media with or without added maltose and/or glucose. The results are shown in Figure 4. The mRNA levels reported were quantified by a phosphorimager and normalized to the levels of actin mRNAs (*ACT1* mRNAs are not shown in Figure 4).

The *MAL62/12* transcript is induced by maltose and repressed by glucose. Disruption of *MIG1* increased the levels of the *MAL62/12* transcript 4-6 fold both in the repressed and induced/repressed media. Additionally, *MAL62/12* mRNA levels are higher in the repressed and induced/repressed conditions than in the uninduced condition in the *mig1* disruption strain. This confirms our finding of a glucose induction of maltase activity in this strain in the absence of *MIG1* (Table 2). As compared to the induced expression level, there also remains a 25-fold glucose repression in induced/repressed cells which is independent of Mig1p. Thus, we conclude that glucose repression of *MAL62/12* can be separated into

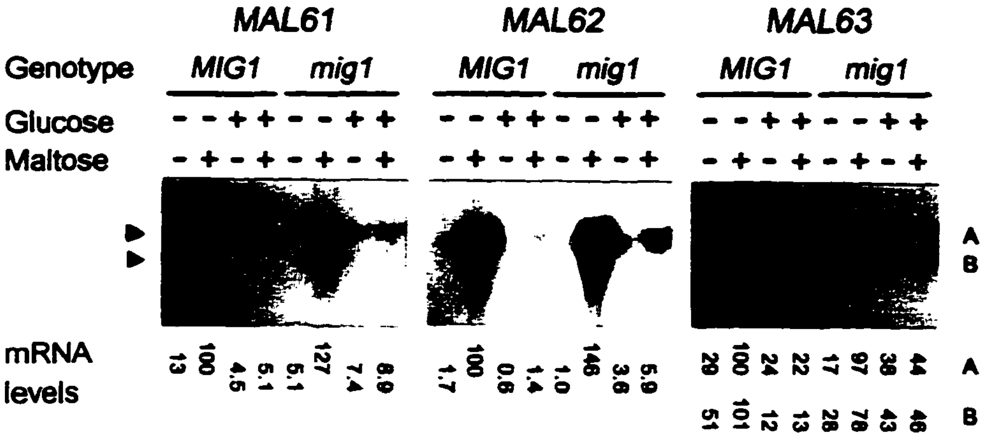


Figure 4. Effect of *mig1* gene disruption on the expression of the *MAL61*, *MAL62/12*, and *MAL63* mRNAs. Strain 332-5A (relevant genotype *MAL61 MAL62 MAL63 mal11 MAL12 mal13*) and 332-5A *mig1Δ* were grown as described in Table 2. Total mRNA was isolated, size-separated using a horizontal agarose gel, and transferred to a nylon membrane which was then hybridized to probes derived from *MAL61*, *MAL62*, and *MAL63* as described in Materials and Methods. The probe used is indicated above each panel. It should be noted that, because of the high level of homology, the *MAL62*-derived probe also detects the *MAL12* mRNA. mRNA levels were quantified in a phosphorimager, and normalized using hybridization to an *ACT1* probe. For each mRNA, the level in wild-type cells grown on maltose was set at 100%. The 2.0-kb mRNA was set at 100% in the case of *MAL63*. The *arrowheads* indicate the positions of the two *MAL61*-homologous mRNAs. The letters *A* and *B* distinguish the two *MAL63*-homologous mRNAs.

at least two mechanisms. One mechanism requires Mig1p and operates both in the presence and in the absence of maltose. The second mechanism is independent of Mig1p and is detected only in the presence of maltose, suggesting that it may involve inhibition of the Mal63p-mediated induction process.

In the case of *MAL61*, low levels of mRNA are detected in uninduced and repressed conditions due to the presence of a transcript which is somewhat larger than the major inducible mRNA (Figure 4; Needleman *et al.*, 1984). The smaller transcript is undetectable under these conditions. Both transcripts respond to maltose induction. The values in Figure 4 are presented as a total of both the inducible and the partially constitutive transcripts. From these results, we are unable to demonstrate an effect of *MIG1* disruption on the expression patterns of either transcript.

MAL63 has two mRNAs of different sizes and both transcripts are induced by maltose (Figure 4; Needleman *et al.*, 1984). Expression levels of the larger transcript are similar under uninduced, repressed and induced/repressed conditions, and disruption of *MIG1* only slightly increased the expression in the presence of glucose. The smaller transcript is sensitive to glucose repression, about four-fold, and *MIG1* disruption leads to an approximately four-fold increase in the levels of this transcript in repressed and induced/repressed conditions. As with *MAL62*, there is also a Mig1p-independent component of repression, which is seen under induced/repressed conditions.

***MIG1*-dependent glucose repression in *MAL*-activator constitutive strains.** As noted above, glucose repression of maltase (*MAL62/12*) mRNA appears to have at least two components: a 4-6-fold repression mediated by Mig1p and a 25-fold Mig1p-independent component which we suggest involves inhibition of maltose induction. To confirm that inhibition of the induction process contributes to glucose repression of the *MAL* genes, we used two *MAL*-activator constitutive alleles. *MAL43-C* is a dominant, constitutive alleles of the *MAL*-activator gene encoded by the *MAL4* locus (Charron and Michels, 1987; Gibson *et al.*, 1997). This gene was introduced into both the *MAL6* strain 332-5A and isogenic *mig1Δ* strain on a *CEN* plasmid. *MAL64* is a linked homologue of *MAL63* which can be activated by nonsense mutation to encode a truncated, dominant constitutive activator of *MAL* gene expression (Dubin *et al.*, 1988; Gibson *et al.*, 1997). Stain R10 is isogenic to 332-5A except for the presence of the constitutive *MAL64-R10* allele and a deletion of *MAL63*. Maltase expression was determined in both the constitutive and isogenic *mig1Δ* strains (Table 3).

Both strains are fully constitutive. Indeed, maltase levels are reproducibly higher in the *MAL64-R10* strain when grown in uninduced rather than induced conditions. This constitutive expression is only repressed approximately ten-fold by glucose, and deletion of *MIG1* causes a three-fold derepression. In the strain carrying the *MAL43-C* plasmid, maltase expression was only three-fold repressed by glucose, and disruption of *MIG1* caused a two-fold derepression (Table 3). Thus, in *mig1Δ* background, the constitutive *MAL*-activators cause

TABLE 3

Effect of a *MIG1* disruption in *MAL*-activator constitutive strains

Strain and genotype	Maltase activity			
	–	Mal	Glu	Mal+Glu
MAL64-R10 strain				
MIG1	1027	752	77	69
mig1Δ	1211	1233	211	174
MAL6 [pMAL43-C] strain				
MIG1	506	561	185	150
mig1Δ	505	732	377	332

The *MAL63-R10* strain, R10, is isogenic to strain 332-5A but carries the constitutive *MAL64-R10* allele and a *mal63::URA3* deletion. This strain and the isogenic *mig1Δ* strain were grown as described in Table 2. To construct the *MAL6* [pMAL43-C], strain 332-5A was transformed with a *CEN* plasmid carrying the *MAL43-C* constitutive *MAL*-activator allele in vector YCp50. This strain and the isogenic *mig1Δ* were grown in minimal media lacking uracil plus 3% glycerol and 2% lactate and 2% of the indicated sugar. Maltase activities were assayed as described in Table 1.

significantly higher levels of maltase expression than the wild-type inducible Mal63p under repressed and induced/repressed conditions. These results confirm that inhibition of the *MAL*-activator-mediated induction process plays a significant role in glucose repression of the *MAL* genes.

Mig1p binds to the upstream regions of *MAL61*, *MAL62*, and *MAL63*. The results reported above suggest that *MIG1* is involved in the glucose regulation of maltose fermentation. The DNA-binding specificity of Mig1p has been documented by extensive analysis demonstrating that the two zinc fingers of Mig1p recognize the degenerate GC-box (G/C)(C/T)GG(G/A)G (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991; Lundin *et al.*, 1994). Previous studies identified binding sites for Mig1p in the promoter regions of *GAL1*, *GAL4* and *SUC2* (Nehlin and Ronne 1990; Nehlin et al. 1991). The Mig1p binding sites in the *GAL1* and *GAL4* promoters lie within regions defined as essential for glucose repression of these genes, referred to as $\mathrm{URS_G}$ for upstream repression sequences (Flick and Johnston, 1990; Flick and Johnston, 1992; Griggs and Johnston, 1991). Deletion analysis of the *MAL61-MAL62* bidirectional promoter region identified a sequence adjacent to the $\mathrm{UAS_{MAL}}$ which appears to be involved in the glucose-repression of *MAL62* and exhibits sequence homology to the Mig1p DNA-binding site consensus sequence (Levine *et al.*, 1992; Lundin *et al.*, 1994). In fact, perusal of the *MAL61-MAL62* intergenic region reveals four sites matching this sequence, which we will refer to as sites A (*MAL61* proximal) to D (*MAL62* proximal) (Figure 5). The *MAL63* promoter contains a single possible Mig1p site approximately 120 basepairs upstream of *MAL63* ORF (Figure 5) (Kim and Michels, 1988).

Agarose gel shifts with end-labeled restriction fragments were used to scan the promoter regions of *MAL61*, *MAL62*, and *MAL63*. The *MAL61-MAL62* intergenic region contains at least two independent Mig1p binding sites, as evidenced from the fact that the non-overlapping fragments 1 and 4 both are retarded in the presence of Mig1 protein (Figure 5). Fragment 4 contains bases from -217 to -371 with respect to the ATG of *MAL62*. The Mig1p-binding region in fragment 1 was further localized using fragments 2 and 3 to a site between basepairs -214 and -417 with respect to the ATG of *MAL61* (Figure 5). For the *MAL63* gene, we found that a fragment which contains DNA from base -196 to +78 is retarded in the presence of Mig1 protein.

We also considered the possibility that the glucose induction of maltase expression in the *mig1* strain could involve the *SKO1* gene which functions as a transcriptional repressor of *SUC2* (Nehlin *et al.*, 1992). The transient glucose induction of *SUC2* in the *mig1* strain seems to be associated with the loss of Sko1p-dependent repression (Nehlin *et al.*, 1992). Therefore, the restriction fragments were also tested for binding to the Sko1 protein. In no case did we detect any retardation (Figure 5). This is consistent with the fact that a deletion of *SKO1* had little effect on *MAL* gene expression (data not shown).

The results in Figure 5 suggest that Mig1p binds to the *MAL63* promoter and to at least two distinct sites in the *MAL61-MAL62* promoter. We used gel-

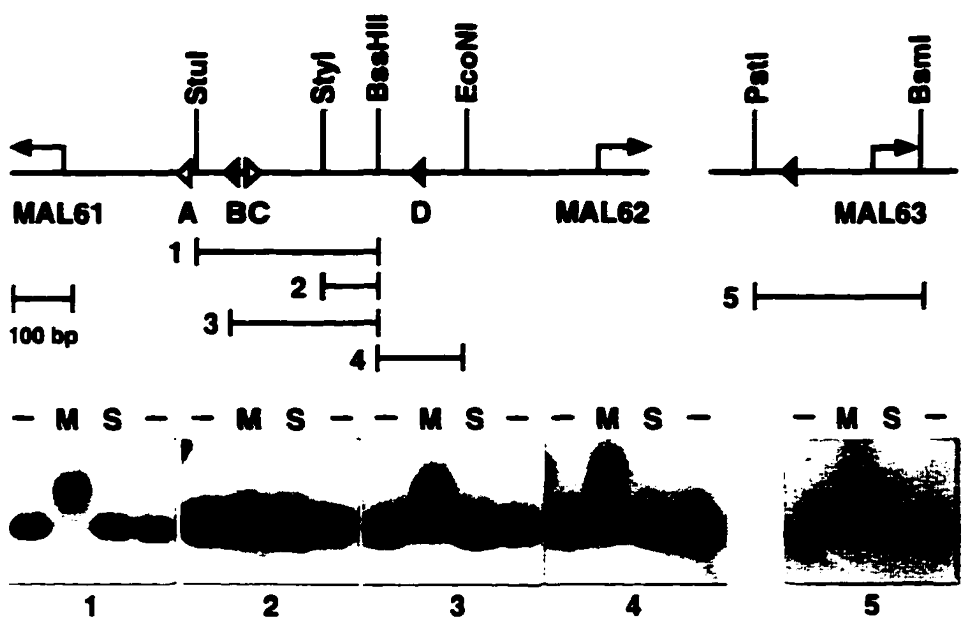


Figure 5. Agarose-gel shift assay for Mig1p-binding to restriction fragments from the *MAL* promoters. Restriction maps of the *MAL61-MAL62* and *MAL63* promoters are shown at the top, with the fragments tested for binding displayed below the maps. Possible Mig1p-binding sites are shown as *arrows*, the direction of which indicates the orientation of the GC-box. *Filled arrows* represent sites to which Mig1p binds strongly. The agarose-gel shifts are shown at the bottom. Abbreviations: *M*, Mig1 protein; *S*, Sko1 protein; -, no added protein. This experiment was carried out by Jan O. Nehlin in Hans Ronne's laboratory.

shift analysis with short double-stranded oligonucleotides corresponding to the various potential Mig1p binding sites identified in the promoter regions of these genes to determine which are capable of binding Mig1p *in vitro*. The results are shown in Figure 6. As a positive control, we also included an oligonucleotide corresponding to the previously identified Mig1p site A in the *SUC2* promoter (Nehlin and Ronne, 1990). We found that the different sites vary considerably in their ability to bind Mig1p. The site in the *MAL63* promoter strongly bound Mig1p, as did site B from the *MAL61-MAL62* region. The *MAL62* proximal site D also bound Mig1p, though less efficiently than site B. Site C bound Mig1p very weakly, which was evident only after prolonged exposure of the gel. The remaining site A did not bind Mig1p at all. These results are consistent with the above data using restriction fragments (Figure 5).

Deletion analysis of the Mig1p binding sites in the *MAL61-MAL62* promoter. To determine whether the Mig1p-binding sites, site B and site D, in the *MAL61-MAL62* identified *in vitro* function *in vivo* and whether Mig1p functions to repress *MAL61*, we monitored the effects of deleting one or both sites on *MAL61* and *MAL62* expression. A 0.9 kilobasepair fragment containing the full 874 basepair *MAL61-MAL62* intergenic promoter sequence and a few codons from the 5'-end of both the *MAL61* and *MAL62* open reading frames was fused, in frame, to the *LacZ* gene of plasmid YCp355, a *CEN* derivative of YIp355 (Levine et al. 1992; Myers et al. 1986). Deletions in which the 6 basepair GC-box was exactly removed from site B (-275 to -280), site D (-580 to -585) and

OLIGO	SITE	SEQUENCE
JN66	MAL63	GTATTAAACCCGGGGTA
JN94	MAL61 A	AGTCTACATCTGGAGAC
JN72	MAL61 B	GTAATTTTTGTGGGGAA
JN90	MAL61 C	GAAAATTATGTGGAGGC
JN96	MAL61 D	GTTAATTGTGTGGGGTC
ML44	SUC2 A	GGTAAAAATGCGGGGAA

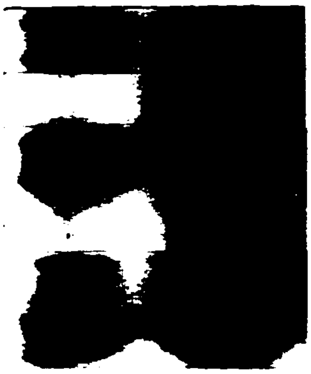


Figure 6. Gel-shift assay for Mig1p-binding to oligonucleotides derived from potential binding sequences found in the *MAL* gene promoters. The top strand of each oligonucleotide is shown to the left, with the GC box *underlined*. The gel shift is oriented with Mig1p-DNA complexes to the left and free DNAs to the right. This experiment was carried out by Jan O. Nehlin in Hans Ronne's laboratory.

from both sites B and D were constructed. The wild-type promoter and the three deletions were fused to the *LacZ* reporter in both the *MAL61* and *MAL62* orientations and these plasmids introduced into the *MAL6* strain 332-5A and an isogenic *mig1* strain. Reporter gene expression was determined in uninduced, induced and repressed conditions (see Table 4).

Expression of *LacZ* from the wild-type *MAL62* promoter in the wild-type *MAL6 MIG1* host strain was induced only 2 to 3-fold, well below the 50 to 100-fold induction seen in measurements of induction based on *MAL62/12* transcript levels (Figure 4) and maltase enzyme levels (Table 2). These results are consistent with our previous findings which used a single, integrated copy of the *LacZ* fusion plasmid (Levine *et al.* 1992). The basis for this apparent decreased inducibility is unclear but it could imply the existence of some as yet undefined regulatory process controlling *MAL62* expression. Nevertheless, it is important to note that the sensitivity to glucose repression exhibited here appears to be comparable to that in the other assay systems.

Expression from the wild-type promoter in the *MAL62-LacZ* orientation was repressed 30- to 80-fold by glucose and deletion of *MIG1* leads to a 10-fold derepression (Table 4). Deletion of the *MAL62*-proximal Mig1p-binding site, site D, relieves glucose repression of *MAL62-LacZ* about 3-fold. Deletion of the *MAL61*-proximal site B alone has no effect on *MAL62-LacZ* expression, but shows a modest effect when site D also is deleted. Deletion of *MIG1* caused only a minor increase in *MAL62-LacZ* expression in the presence of glucose when

TABLE 4

Effect of deletion of the two Mig1p-binding sites in the *MAL61-62* promoter on a *LacZ* reporter gene

Promoter	β-Galactosidase activity							
sequence	*MAL6 MIG1* strain				*MAL6 mig1Δ* strain			
orientation	–	Mal	Glu	Mal+Glu	–	Mal	Glu	Mal+Glu
MAL61-LacZ								
Wild-type	2	18	<1	<1	2	35	<1	1
ΔB	2	17	<1	<1	2	19	1	2
ΔD	1	30	<1	<1	2	40	1	2
ΔB, ΔD	<1	16	<1	<1	<1	24	1	2
MAL62-LacZ								
Wild-type	69	170	2	2	60	200	20	22
ΔB	50	169	2	3	48	212	20	27
ΔD	16	101	6	6	14	124	19	21
ΔB, ΔD	14	122	9	9	16	155	19	21

Mutations in the 874 basepair *MAL61-62* bidirectional promoter sequence, deleting one or both of the G/C-boxes of the two Mig1p-binding sites, were constructed and fused, in-frame, to the *LacZ* gene of plasmid YCp355 (see Materials and Methods) in both the *MAL61* and *MAL62* promoter orientations. These were transformed into strain 332-5A and an isogenic *migΔ*. Cells were grown in minimal media lacking uracil plus 3% glycerol and 2% lactate and 2% of the indicated sugar. β–Galactosidase activities were assayed in permeabilized cells and the activity was normalized to cell density (expressed as Miller units).

both Mig1p sites had been deleted (Table 4). This demonstrates that the effect of Mig1p on *MAL62* expression is mediated almost entirely by the Mig1p-binding sites.

Interestingly, uninduced expression from the *MAL62* promoter was significantly reduced as a result of deletion of site D. Loss of Mig1p had no effect on this uninduced expression. This suggests that an activator protein also may bind to site D (see discussion).

Unfortunately, *LacZ* expression from the *MAL61* promoter is surprisingly low even under induced conditions and maltose induction of the *MAL61-LacZ* fusion is only about 10-fold (Table 4). In an effort to examine the effect of *MIG1* on *MAL61*, we decided to test the promoter deletions in the *MAL64-R10* constitutive strain. All eight *LacZ* fusion constructions were introduced into strain R10u which is isogenic to strain R10 described above except for a *ura3* mutation selected in the *mal63::URA3* disruption using 5-FOA. Expression of *MAL61-LacZ* is now adequate to allow us to evaluate the effects of deletions of sites B and D. As has been noted previously in constitutive strains, the uninduced levels of expression are higher than those under induced conditions (Tables 3 and 5; Dubin *et al.*, 1988). Table 5 shows a 14-fold glucose repression of *MAL61-LacZ* expression for the wild-type promoter sequence. Deletion of site B relieves glucose repression of *MAL61-LacZ* expression two-fold. Deletion of site D slightly increases expression under all conditions but the 14-fold glucose sensitivity remains. The double deletion results in a three-fold increase in

TABLE 5

Effect of deletion of Mig1p-binding sites in the *MAL61-62* promoter on *LacZ* reporter gene expression in a *MAL64-R10* constitutive strain

Promoter orientation	β-Galactosidase activity			
	–	Mal	Glu	Mal+Glu
MAL61-LacZ				
Wild-type	110	40	8	8
ΔB	123	49	16	16
ΔD	189	53	13	15
ΔB, ΔD	90	34	21	21
MAL62-LacZ				
Wild-type	387	224	85	88
ΔB	369	229	105	108
ΔD	244	193	122	121
ΔB, ΔD	388	201	154	151

Plasmids carrying the *MAL61-LacZ* and *MAL62-LacZ* fusion genes described in Table 4 were introduced into strain R10u, a uracil-requiring spontaneous mutant isolated from strain R10 using 5-FOA selection. The cells were grown and β-galactosidase levels assayed as described in Table 4.

glucose-repressed expression. Thus, glucose repression of *MAL61* is mediated by site B, and site D does not contribute significantly to the glucose repression of *MAL61*. The effects of glucose on the expression of the *MAL62-LacZ* fusions in the constitutive strain are consistent with those seen in the wild-type strain.

The wild-type *LacZ* fusions also were introduced into a *mig1Δ* of strain R10u and *LacZ* expression determined (Table 6). Induced expression of both *MAL61* and *MAL62* was increased to levels comparable to the uninduced levels. We found that glucose repression of *MAL61* and *MAL62* was relieved 5-fold and 2.5-fold, respectively. An approximately 2-fold glucose repression remains for both genes. These results are consistent with those reported in Table 3 where maltase levels were determined in a *MAL64-R10* constitutive strain and confirm that Mig1p is involved in glucose repression of *MAL61* as well as *MAL62*.

Sensitivity of a *MAL63/43* hybrid gene to glucose repression. The results reported above indicate that glucose negatively regulates the maltose-induced transcriptional activity of Mal63p in two ways: a Mig1p-mediated repression of *MAL63* transcription (Figure 4) and a Mig1p-independent inhibition of the Mal63p function (Table 2 and 4; Figure 4). To analyze the significance of the repression by Mig1p relative to the inhibition of maltose induction on *MAL* gene expression, a plasmid carrying a *MAL63* or a *MAL63/43-C* hybrid gene was transformed into strain YPH500 and an isogenic *mig1Δ* strain. The wild-type strain YPH500 contains the *MAL* structural genes but no *MAL*-activator gene. The *MAL63/43-C* hybrid gene, in which the C-terminal regulatory domain of the

TABLE 6

Effect of a *MIG1* disruption on *LacZ* reporter gene expression in a *MAL64-R10* constitutive strain

Promoter sequence orientation	β-Galactosidase activity							
	MAL64-R10 MIG1 strain				*MAL64-R10 mig1Δ* strain			
	–	Mal	Glu	Mal+Glu	–	Mal	Glu	Mal+Glu
MAL61-LacZ	110	40	8	8	97	109	48	41
MAL62-LacZ	387	224	85	88	372	413	212	198

A *mig1Δ* null mutation was introduced into strain R10u as descibed in Table 4 above. Plasmids carrying the *MAL61-LacZ* and *MAL62-LacZ* fusions with the wild-type promoter sequence were introduced into strain R10u and the isogenic *mig1Δ* strain, and β-galactosidase activities determined as described in Table 4.

constitutive *MAL43-C* is fused to the N-terminal portion of the maltose inducible *MAL63*, is under the control of the *MAL63* promoter but is fully constitutive thereby bypassing the requirement for maltose to activate *MAL* gene transcription including its own (Gibson *et al.*, 1997). Strains carrying both plasmids were grown in glucose media and maltase activities determined. *MAL63/43-C* is still sensitive to glucose repression in wild-type strain YPH500 (Table 7) despite the fact that it does not require maltose for its transcriptional activity (Gibson *et al.*, 1997). Disruption of *MIG1* significantly increases maltase expression by about 70-fold. The magnitude of this increase is much larger than in *mig1Δ* cells carrying an inducible *MAL63* which derepresses maltase expression six-fold. The constitutive *MAL43* lacking the Mig1p-binding site in its promoter exhibits glucose insensitivity in activating maltase expression in wild-type *MIG1* strains (Charron and Michels, 1987; Gibson *et al.*, 1997; see Table 7). Thus, Mig1p repressor effectively counteracts the activation by Mal63/43-Cp on the *MAL63* promoter in response to glucose.

The effects of two other glucose repression-resistant mutants, *hxk2* and *reg1*, on glucose sensitivity of strains carrying *MAL63* and *MAL63/43-C* also were examined and compared to that of *mig1* (Table 7). Both *REG1* and *HXK2* are thought to function upstream of Snf1 kinase in glucose signal transduction pathway and therefore upstream of Mig1p (Johnston and Carlson, 1992). Like *mig1Δ*, deletion of *REG1* causes a dramatic loss of glucose sensitivity of *MAL63/43-C*. However, disruption of *HXK2* derepresses maltase expression only

TABLE 7

Glucose repression in various mutants carrying *MAL63* or *MAL63/43-C* hybrid genes

Relevant genotype	Maltase Activity *MAL63*	Maltase Activity *MAL63/43-C*
Wild type	0.8	3.7
mig1Δ	4.7	271
hxk2Δ	26	113
mig1Δ hxk2Δ	36	446
reg1Δ	23	550

MIG1 and *HXK2* were disrupted in strain YPH500. Strain DF186 carries a *reg1::URA3* null allele. All these strains were transformed with plasmids pUN30/MAL63 or pUN30/MAL63-43C (Gibson et al., 1997). Cells were grown in synthetic media minus tryptophan with 2% glucose and maltase activities assayed as described in Table 1.

about 30-fold in both cells carrying *MAL63* and cells carrying *MAL63/43* and thus is significantly less effective than loss of Reg1p or Mig1p. A *mig1Δ hxk2Δ* double disruption appears to have a cumulative effect over either disruption alone on the glucose sensitivity of *MAL63/43-C*, and produces an effect similar to that of *reg1Δ*.

Part II. Glucose inhibition of the *MAL*-activator-mediated maltose induction

Overexpression of the *MAL*-activator does not relieve glucose inhibition. The results reported above indicate that the function of Mal63p as a maltose-inducible transactivator is inhibited by glucose independent of the effects of Mig1p. We define "glucose inhibition" as those processes that affect *MAL*-activator-mediated maltose induction in order to distinguish them from glucose repression effects which act more directly on the *MAL* gene promoters. Since glucose could inhibit maltose induction by affecting the synthesis and/or stability of the Mal63 protein, we decided to test whether overexpression of *MAL63* could abolish glucose inhibition. A multicopy *LexA-MAL63* fusion plasmid was introduced into a *mig1Δ* strain carrying a *lexA-LacZ* reporter gene. A *mig1Δ* strain was used here in order to eliminate the repressing effect of Mig1p thereby allowing the examination of glucose sensitivity of the *MAL*-activator. The *LexA-MAL63* fusion gene, in which the full-length *MAL63* ORF is fused to codons 1-87 of *E.coli LexA*, was overexpressed from the constitutive *ADH1* promoter and able to activate transcription of both *MAL* structural genes and the *lexA-LacZ* reporter gene in a maltose-dependent manner (Gibson, 1995; Table 8 and 9). Western analysis of whole cell extracts using anti-LexA antibody showed that the LexA-Mal63 fusion protein was expressed at comparable levels in both maltose-grown and glucose-grown cells which were about twice as much as the level in the glycerol/lactate-grown cells (data not shown). As shown in Table 8, the LexA-

TABLE 8

Glucose inhibition of the overexpressed LexA-Mal63 protein

Relevant genotype	Expressed protein	β-Galactosidase activity			Maltase activity		
		–	Mal	Mal+Glu	–	Mal	Mal+Glu
mig1Δ	LexA-MAL63	2	155	2	46	1800	75
	LexA-MAL63$_{(\Delta 41\text{-}66)}$	3	646	ND	16	38	ND
	LexA-MAL63; MAL64-R10	2	186	5	2283	3421	1042
mig1Δ hxk2Δ	LexA-MAL63	1	398	116	65	1812	1048

The *lexA-LacZ* reporter plasmid used was pSH18-18. *MIG1* and *HXK2* were disrupted in strain YPH500. The *LexA-MAL63* fusion is carried on a 2μ plasmid with *TRP1* (see Materials and Methods) and is able to complement the *MAL*-activator null mutation. The *MAL63*$_{(\Delta 41\text{-}66)}$ mutant allele was fused to *LexA*$_{(1\text{-}87)}$ (pSH2-1) (Gibson, 1995). The constitutive *MAL64-R10* gene is carried on the *HIS3* vector pRS413 (Medintz *et al.*, 1996). Cells were grown in synthetic media lacking uracil, tryptophan and/or histidine containing 3% glycerol and 2% lactate (v/v) plus 2% of the specified sugar (w/v). Both activities were normalized to the protein concentration of the cell extracts. Values are averages of two-four independent transformants assayed in duplicate. Standard errors were less than 10% for maltase activities and usually less than 20% for β-galactosidase activities. ND, not determined.

Mal63 fusion protein failed to activate both the *LacZ* reporter gene and the maltase gene in cells grown in maltose/glucose media. Maltase activity was only two-fold higher than in the glucose-grown cells (34 units, not shown in Table 8). These results indicate that cells which overproduce LexA-Mal63 protein are still subject to glucose inhibition of maltose induction and suggest that the effect(s) of glucose on *MAL*-activator function is not the result of reduced levels of the activator protein.

Inducer exclusion is not necessary for glucose inhibition. It has been demonstrated that maltose induction requires the presence of a functional maltose permease gene (Charron *et al.*, 1986). Disruption of the maltose permease gene eliminates maltose-induced expression of maltase while disruption of the maltase gene has no effect on expression of maltose permease (Charron *et al.*, 1986; Dubin, 1987). Glucose causes a rapid inactivation of maltose permease (Medintz *et al.*, 1996). These findings raise the possibility that glucose may block the induction signal by simply inactivating the maltose permease (inducer exclusion). Two experiments were carried out to explore this hypothesis.

First, to test the minimal level of maltose permease activity required for induction, a *LexA-MAL63*$_{(\Delta 41\text{-}66)}$ fusion gene was transformed into a *mig1Δ* strain. This fusion protein lacks the residues immediately adjacent to the N-terminal DNA-binding zinc cluster of Mal63p. The corresponding region in several other yeast transcription activators with homologous zinc cluster plays an important role in sequence-specific DNA binding (Reece and Ptashne, 1993). This region could have a similar role in Mal63p since the LexA-MAL63$_{(\Delta 41\text{-}66)}$ fusion is unable to activate the expression of the *MAL* structural genes (Gibson, 1995; Table 8 and 9). Maltose permease activity remains at its uninduced basal level in the presence of maltose (Table 9). Despite this low level of maltose permease activity, LexA-Mal63$_{(\Delta 41\text{-}66)}$ is able to activate the transcription of the *lexA-LacZ* reporter gene in response to maltose (Table 8), suggesting that the low basal level expression of maltose permease is adequate for maltose induction.

Second, a plasmid carrying a constitutive *MAL*-activator gene, *MAL64-R10*, was introduced into a *mig1Δ* strain along with the *LexA-MAL63* fusion. *MAL64-R10* is both constitutive and glucose repression insensitive (Dubin *et al.*, 1988; see Table 3). Transformants carrying both the *LexA-MAL63* and *MAL64-R10* genes expressed the *MAL* structural genes even in the presence of glucose (Table 8 and 9). Nevertheless, despite the fact that maltose permease activity in the maltose/glucose-grown cells is increased significantly above basal level, the overexpressed inducible LexA-Mal63 fusion protein is unable to activate transcription of the *lexA-LacZ* reporter (Table 8). Thus, elimination of both inducer exclusion and down-regulation of Mal63 protein levels is not sufficient to relieve glucose inhibition, indicating that an additional mechanism is the major contributor to the Mig1p-independent inhibition of the *MAL*-activator.

***HXK2* is involved in glucose inhibition of Mal63p in addition to normal glucose repression.** The preceding evidence suggests that glucose

TABLE 9

Maltose permease activity in cells with the overexpressed LexA-Mal63 fusion proteins

Expressed protein	Maltose transport activity –	 Mal	 Mal+Glu
LexA-MAL63	1.2	36.9	<0.5
LexA-MAL63$_{(\Delta 41\text{-}66)}$	1.0	1.0	ND
LexA-MAL63; MAL64-R10	171	53.6	3.7

The *LexA-MAL63* fusion plasmids and pRS413/MAL64-R10 were transformed into a *mig1Δ* strain. Cells were grown as described in Table 8. Maltose permease activities were measured as described in Materials and Methods and are expressed as nmol ^{14}C-maltose/mg dry wt./10 min. ND, not determined.

may act upon the maltose induction process and/or on events required for induction process (induction-dependent mechanism). Mutations in the *HXK2* gene relieve glucose repression of many glucose-repressible genes (reviewed in Johnston and Carlson, 1992). *HXK2* has been proposed to be an upstream regulator of two glucose signal transduction pathways: the Snf1 protein kinase pathway and the Snf3/Rgt2-Grr1 pathway which is used to monitor the extracellular glucose (reviewed in Johnston and Carlson, 1992; Ozcan and Johnston, 1995). To test for the involvement of *HXK2* in the glucose inhibition of Mal63p, the *HXK2* gene was disrupted in a *mig1Δ* strain carrying a *lexA-LacZ* reporter. *LexA-MAL63* when introduced into the *mig1Δ hxk2Δ* strain is able to activate both maltase and *LacZ* reporter genes dramatically in the presence of glucose plus maltose (Table 8). This result, together with those described above excluding inducer exclusion as a major mechanism of glucose inhibition of *LexA-MAL63*, suggests that *HXK2* is required for glucose to inhibit Mal63p function through an induction-dependent mechanism.

Functional relationship between Hxk2p and Mig1p in glucose repression. The transcription of *MAL63*, as well as the *MAL* structural genes, is repressed in the presence of glucose by Mig1p (see Figure 4). Mig1p was identified as a downstream factor of the Snf1p signal transduction pathway (reviewed in Johnston and Carlson, 1992; Trumbly, 1992; Ronne 1995). Since *HXK2* was thought to function at an earlier stage of glucose regulatory pathway (reviewed in Johnston and Carlson, 1992), one would expect that disruption of

HXK2 would eliminate both the Mig1p-dependent transcriptional repression of *MAL63* and the *MAL* structural genes and the Mig1p-independent inhibition of Mal63p. To test this assumption, a plasmid carrying a wild type *MAL63* gene was introduced into strain YPH500 and isogenic *mig1Δ*, *hxk2Δ* strains and maltase activities determined. Disruption of *MIG1* or *HXK2* individually relieves glucose repression of maltase gene in glucose-grown cells though to different extents (Table 10). Surprisingly, maltase activity in the maltose/glucose-grown *hxk2Δ* cells is about three-fold higher than the level in glucose-grown cells but still some six-fold lower than the level in maltose-grown cells. This somewhat unexpected result led us to speculate that even in the *hxk2Δ* background Mig1p might still be able to repress *MAL* gene transcription, particularly that of *MAL63*. Consistent with this hypothesis is the observation that maltase activity in *hxk2Δ* cells carrying the overexpressed *LexA-MAL63* fusion gene and grown in maltose/glucose medium is nearly as high as in cells grown in maltose medium (Table 10). To test this repression effect by Mig1p, *MAL63* was introduced into a *mig1Δ hxk2Δ* strain and maltase activities assayed (Table 10). Disruption of *HXK2* in the *mig1Δ* background significantly increases the maltase activity expressed in the presence of glucose plus maltose (Table 10). It appears from these results that Mig1p is still able to repress the expression of *MAL* genes, especially *MAL63*, in the absence of Hxk2p. Since *MAL63* is autoregulated (Needleman *et al.*, 1984; Figure 4), its transcriptional repression by *MIG1* is

TABLE 10

Effect of *HXK2* disruption on glucose inhibition of Mal63 protein

Expressed Mal-activator	Relevant genotype		Maltase activity		
		–	Mal	Glu	Mal+Glu
MAL63	Wild type	26	742	0.8	1.0
	mig1Δ	29	974	4.7	5.2
	hxk2Δ	22	433	26	72
	mig1Δ hxk2Δ	27	477	36	277
LexA-MAL63	*hxk2Δ*	73	1993	80	1495

YPH500 and isogenic mutant strains were transformed with plasmids pUN30/MAL63 (Danzi, 1997) and pLexA/MAL63 carrying *MAL63*, *LexA-MAL63*, respectively. Cells were grown in minimum media lacking tryptophan with 3% glycerol and 2% lactate plus 2% of various specified sugars. Maltase activities were assayed as described in Table 1.

expected to have a significant negative impact on maltase expression in the presence of glucose plus maltose.

The Mig1p-binding site located in the *MAL63* promoter is not present in the promoters of *MAL23* and *MAL43-C* due to a loss of a single base pair in the crucial GC-box (Gibson *et al* ., 1997). The constitutive *MAL23* mutants and *MAL43-C* are glucose insensitive while the constitutive *MAL63* mutants maintaining the Mig1p binding site in their promoters are still sensitive to glucose (Gibson *et al.*, 1997; Wang and Needleman, 1996). To test for the glucose sensitivity of *MAL23*, a plasmid carrying *MAL23* was introduced into YPH500 and isogenic *mig1Δ*, *hxk2Δ* strains. Assays of maltase activities revealed that *MAL23* is partially active in wild type and *mig1Δ* cells grown in maltose/glucose media (Table 11). In contrast to *MAL63*, disruption of *HXK2* in YPH500 caused a complete loss of glucose sensitivity of *MAL23* in maltose/glucose-grown cells. A plasmid carrying the *MAL63/23* hybrid gene in which the C-terminal portion of *MAL23* was fused to N-terminal part of *MAL63* with a *MAL63* promoter and functions as a maltose-inducible activator (Danzi, 1997) was also transformed into a *mig1Δ* strain and maltase activities assayed. The inducible *MAL63/23* was found to be completely sensitive to glucose in *mig1Δ* strain (data not shown), suggesting that the C-terminal regulatory domain of *MAL23* is not sufficient to cause partial glucose insensitivity of *MAL23*. Nevertheless, these findings support the view that, in the *hxk2Δ* strain, Mig1p manages to confer glucose sensitivity on *MAL63* by repressing its transcription, and is able to receive a

TABLE 11

Hexokinase-mediated glucose effects in strains carrying *MAL23* or *MAL13*

Mal-activator allele	Relevant genotype	Maltase Activity –	 Mal	 Glu	 Mal+Glu
MAL23	Wild type	65	1389	1.3	84
	mig1Δ	63	1502	14	105
	hxk2Δ	40	2855	39	2768
MAL13	Wild type	27	832	0.1	0.3
	hxk2Δ	24	802	5.8	160
	hxk1Δ hxk2Δ	23	1415	34	1153

Cells of CMY1001 (*HXK2*), CMY1006 (*hxk2::URA3*) and CMY1015 (*hxk1::TRP1 hxk2::URA3*) carrying *MAL13* at *MAL1* locus (Medintz et al., 1996; Jiang, 1997) were grown in rich media with glycerol and lactate plus various sugars as specified. Plasmid pUN30/MAL23 was transformed into YPH500 and isogenic *mig1Δ*, *hxk2Δ* strains. Cells were grown and maltase activities assayed as described in Table 10.

glucose signal through an Hxk2p-independent pathway. We suggest that, in *hxk2Δ* background, another sugar kinase may at least partially substitute for the role of hexokinase PII encoded by *HXK2* in mediating glucose repression (addressed below in the following section).

***HXK1* may partially mediate glucose repression in a *hxk2Δ* strain.** Strain CMY1001 is congenic to YPH500 and contains a *MAL13* gene at *MAL1* locus (Medintz *et al.*, 1996). Strain CMY1006 is isogenic to CMY1001 except for a *hxk2::URA3* disruption (Medintz *et al.*, 1996). Assays of maltase levels in CMY1001 showed that *MAL13* is completely sensitive to glucose repression (Table 11). Disruption of *HXK2* in CMY1001 relieves glucose repression of maltase expression in glucose-grown cells and significantly increases maltase level in maltose/glucose-grown cells (Table 11). A plasmid carrying the *MAL61-62* promoter fused, in either *MAL61* or *MAL62* orientation, to the *LacZ* gene was transformed into CMY1001 and CMY1006u, a *ura3* derivative of CMY1006 isolated from 5-FOA selection, and β-galactosidase activities determined. The results shown in Table 12 confirm that the relief of glucose repression of *MAL* structural genes by *hxk2Δ* occurs at the transcriptional level. The effect of the loss of Hxk2p on the Mal13 activator is more similar to that seen for Mal63p than the effect with Mal23p where complete insensitivity to glucose is demonstrated in glucose/maltose-grown cells.

Maltase activity in the absence of Hxk2p is still five-fold lower in maltose/glucose-grown cells than in maltose-grown cells, suggesting the

TABLE 12

Effect of a *HXK2* disruption on *LacZ* reporter gene expression

Promoter sequence orientation	β-Galactosidase activity							
	HXK2 strain				*hxk2Δ* strain			
	–	Mal	Glu	Mal+Glu	–	Mal	Glu	Mal+Glu
MAL61-LacZ	4	42	<1	<1	5	23	<1	3
MAL62-LacZ	47	239	1	2	50	174	4	31

Plasmids carrying the *MAL61-LacZ* and *MAL62-LacZ* fusions were introduced into strain CMY1001 (*HXK2*) and CMY1006u (*hxk2Δ*) which is a *ura3* derivative of CMY1006 isolated on 5-FOA medium. Cells were grown as described in Table 4. β-Galactosidase activities were assayed in permeabilized cells and expressed as Miller units.

existence of a residual glucose repression effect even in the *hxk2Δ* strain (Table 11). *S. cerevisiae* contains two homologous hexokinases, PI (Hxk1p) and PII (Hxk2p) (Lobo and Maitra, 1977). *HXK2* appears to be expressed on various carbon sources while expression of *HXK1* is glucose repressed (Herrero *et al.*, 1995; De Winde *et al.*, 1996). *HXK2* is required to mediate glucose repression of *HXK1* (De Winde *et al.*, 1996). The functional distinction between *HXK1* and *HXK2* appears to be the result of their different expression patterns since multiple copies of *HXK1* in an *hxk2* mutant partially restore glucose repression (Entian *et al.*, 1984; Ma and Botstein, 1986; Rose *et al.*, 1991). Since *HXK1* is derepressed in *hxk2Δ* cells on glucose, we reasoned that Hxk1p could play some of the roles of Hxk2p in generating/transmitting glucose repression signal. To test this hypothesis, *HXK1* was disrupted in CMY1001 and CMY1006 (*hxk2Δ*). Maltase expression on various carbon sources was not affected by disruption of *HXK1* alone (data not shown). Assays of maltase activities in *hxk1Δ hxk2Δ* strain showed that disruption of *HXK1* in *hxk2* background further relieved glucose repression of maltase in glucose-grown cells (Table 11). More significantly, glucose sensitivity of *MAL13* was almost completely abolished in maltose/glucose-grown cells. These data suggest that Hxk1p, in *hxk2* background, can at least partially substitute for Hxk2p in mediating glucose repression.

Glucose-induced inactivation of maltose permease is intact in the *hxk2Δ* strain. Strain CMY1001 contains an HA-tagged maltose permease gene, *MAL61/HA*, in addition to *MAL12* and *MAL13* (Medintz *et al.*, 1996). Maltose transport activity and the amount of maltose permease protein can be followed in this strain to assay the sensitivity of permease to glucose-induced inactivation. The protocol for inactivation assay has been described in detail in Medintz *et al.* (1996). Cells were grown to early log phase in rich medium containing 2% maltose (to induce maltose permease expression), harvested and transferred to nitrogen-starvation medium (to stop protein synthesis) with 2% glucose. Maltose transport activity and maltose permease protein levels are measured at selected time intervals within 3 hours following the transfer to glucose.

Glucose causes a decrease in both maltose transport activities and the protein levels of maltose permease (Figure 7, Panel A). Disruption of *HXK2* in CMY1001 has little effect on the glucose inactivation of maltose permease (Figure 7, Panel B). The *hxkΔ* cells pregrown in maltose/glucose medium with partially induced expression of maltose permease also show a complete inactivation after being transferred into glucose medium (Figure 7, Panel C). These results suggests that glucose-induced inactivation of maltose permease alone is not sufficient to block the maltose induction signal since *hxk2Δ* relieves glucose inhibition of the *MAL*-activator function (Table 11).

Mutation in *REG1* relieves both general glucose repression of *MAL* genes and glucose inhibition of Mal63p. *REG1* encodes a regulatory subunit of protein phosphatase type-1 (PP1) (Tu and Carlson, 1995). PP1 and associated Reg1p are required for glucose repression and act upstream of Snf1

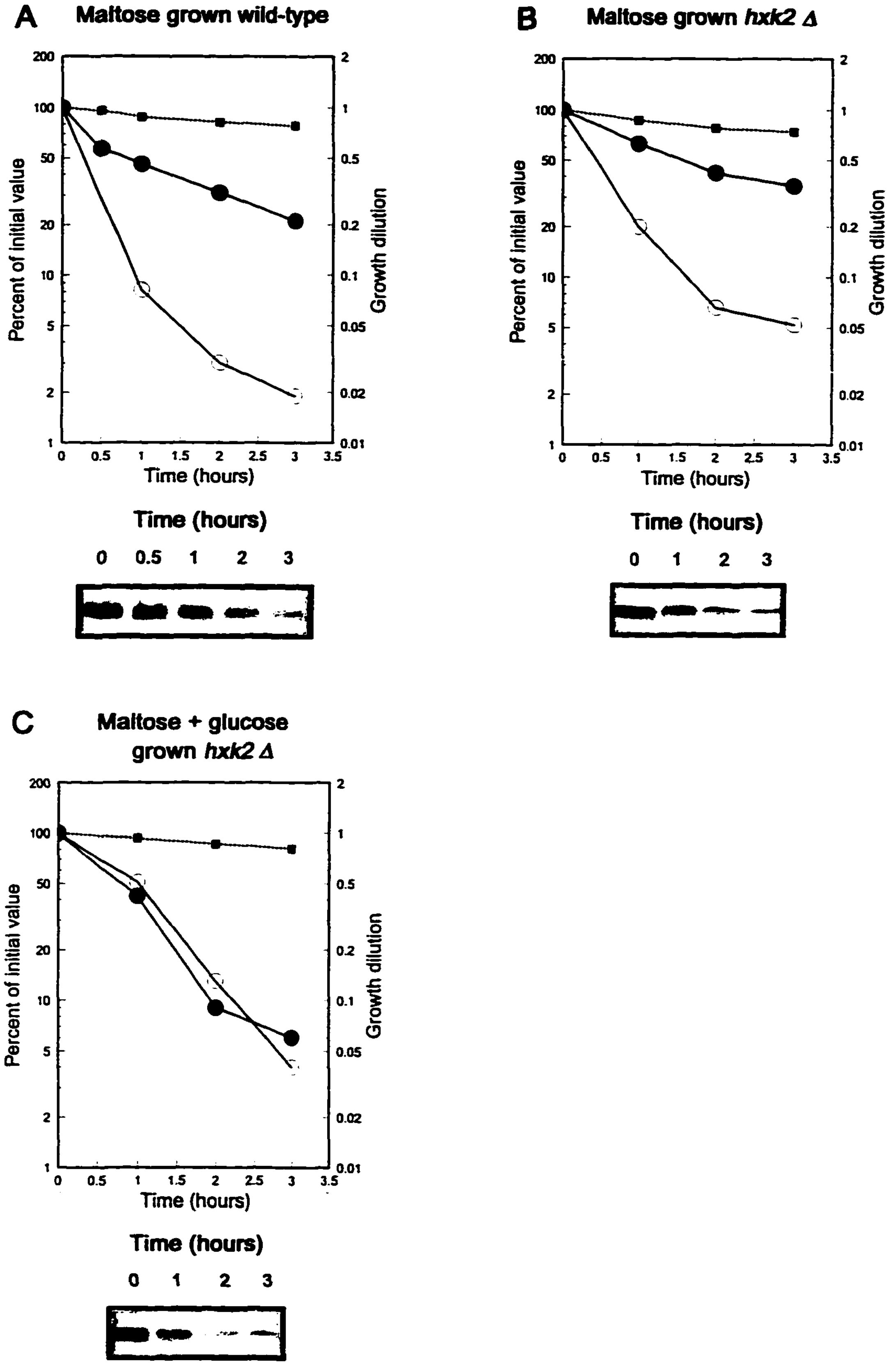

A
Maltose grown wild-type
Percent of initial value
Growth dilution
200
100
50
20
10
5
2
1
2
1
0.5
0.2
0.1
0.05
0.02
0.01
0
0.5
1
1.5
2
2.5
3
3.5
Time (hours)
Time (hours)
0 0.5 1 2 3
B
Maltose grown hxk2 Δ
Percent of initial value
Growth dilution
Time (hours)
Time (hours)
0 1 2 3
C
Maltose + glucose grown hxk2 Δ
Percent of initial value
Growth dilution
Time (hours)
Time (hours)
0 1 2 3

Figure 7. Glucose-induced inactivation of maltose permease in the *hxk2Δ* strain. Strains CMY1001 (wild-type) and CMY1006 (*hxk2Δ*) were grown in rich media containing 2% maltose (Panels A and B) or 2% maltose plus 2% glucose (Panel C) to early log phase (OD_{600} of 0.2), and the standard inactivation assay protocol was used as described in Medintz *et al.*, 1996. The relative levels of Mal61/HA protein (•), maltose transport activity (o) and growth dilution (▪) are plotted in the same panel. The relative protein level and transport activity at time X are compared to the corresponding values at time zero. Growth dilution is calculated as the OD_{600} at time zero divided by the OD_{600} at time X. Representative Western blots are shown at the bottom. Equal amount of protein (about 80-100μg) were loaded into each lane of a 10% SDS-polyacrylamide gel. After transfer, membranes were probed with anti-HA antibody (Boehringer Mannheim) and horseradish peroxidase-linked sheep anti-mouse antibody. Protein blots were visualized with the ECL Western blot kit (Amersham) on ECL-Hyperfilm and quantified by scanning with a Beckman DU640 spectrophotometer (Medintz *et al.*, 1996). The inactivation assay was carried out by Hua Jiang in our laboratory.

kinase (Tu and Carlson, 1996). To explore the role of *REG1* in glucose regulation of *MAL* genes, a *MAL63* plasmid was transformed into the wild type strain KT1357 and isogenic *reg1Δ* strain DF186. Deletion of *REG1* relieves repression of maltase in glucose-grown cells (Table 13). More significantly, *reg1* mutation increased maltase activity dramatically in maltose/glucose-grown cells, indicating a maltose-induced activation of Mal63p. As previously shown in Part I, the constitutive *MAL63/43-C* hybrid gene introduced into the *reg1Δ* strain, as in the *mig1Δ hxk2Δ* strain, also lost its sensitivity to glucose (see Table 7). Thus, in contrast to *hxk2Δ*, disruption of *REG1* appears to relieve the Mig1p-dependent glucose repression of *MAL* genes as well as the Mig1p-independent glucose inhibition of Mal63p through induction-dependent mechanism.

***GRR1*-dependent pathway mediates inhibition of the *MAL*-activator function in response to glucose.** Mutations in *RGT1* suppress the defects in high-affinity glucose transport and glucose repression caused by *grr1* mutations (Vallier *et al.*, 1994; Erickson and Johnston, 1994; Ozcan and Johnston, 1995). *RGT1* encodes a zinc cluster protein which negatively regulates *HXT* expression (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996). Grr1p is required for inhibition of Rgt1p in response to glucose (Ozcan *et al.*, 1996). Since *grr1Δ* is defective in glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997) as well as glucose repression, we intended to examine whether *grr1Δ* also relieves induction-dependent glucose inhibition of the *MAL*-activator. As shown in Table 14, disruption of *GRR1* in strain CMY1001 relieves glucose repression of

TABLE 13

Effect of a *reg1* disruption on glucose inhibition of *MAL63*

Relevant genotype	Maltase activity –	 Mal	 Glu	 Mal+Glu
REG1	18	781	1.0	1.3
reg1Δ	17	814	23	331

Plasmid pUN30/MAL63 was transformed into strains KT1357 (*REG1*) and isogenic DF186 (*reg1Δ*). Cells were grown and maltase activities measured as described in Table 10.

maltase expression in glucose-grown cells and abolished glucose sensitivity of *MAL13* in maltose/glucose-grown cells, indicating a loss of the induction-dependent glucose effect. Disruption of *RGT1* in the *grr1Δ* strain dramatically restores glucose repression in cells grown in glucose medium (Table 14), which correlates well with the full restoration of high-affinity glucose transport in the same *grr1Δ rgt1Δ* strain (Jiang *et al.*, 1997). However, glucose sensitivity of *MAL13* is only partially restored and an induced expression of maltase gene is still seen in maltose/glucose-grown cells. Maltase expression in a *grr1Δ rgt1Δ* strain carrying an overexpressed *LexA-MAL63* fusion gene also is clearly induced in maltose/glucose-grown cells as compared to glucose-repressed level (Table 14). These results suggest a Grr1p-dependent glucose effect on *MAL*-activator induction which does not involve Rgt1p.

Rgt2p and Snf3p appear to function as sensors of high and low extracellular glucose, respectively, generating a signal to activate expression of *HXT* genes, via Grr1p (Ozcan and Johnston, 1995; Ozcan *et al.*, 1996). *RGT2-1* is a dominant mutation which produces a constitutive glucose signal to induce *HXT1* expression (Ozcan *et al.*, 1996). If a *GRR1*-dependent glucose inhibition of Mal13p function requires the high-glucose sensor Rgt2p, maltase expression in a strain carrying an *RGT2-1* allele would be affected even under maltose-induced conditions. To test this idea, a plasmid carrying *RGT2*-1 was introduced into CMY1001 and maltase activities under uninduced and induced conditions were assayed. Maltose-induced expression of maltase is only slightly affected by

Table 14

GRR1-dependent glucose inhibition of *MAL*-activator

Relevant genotype	Maltase activity		
	Glu	Mal+Glu	Mal
Wild type	0.1	0.3	832
grr1Δ	13	1197	1691
grr1Δ rgt1Δ	1.4	49	619
grr1Δ rgt1Δ [pLexA-MAL63]	3.0	142	ND
rgt2Δ	0.2	2.6	447

GRR1 and *RGT2* were disrupted in wild-type strain CMY1001 carrying a *MAL13* gene (Jiang *et al.*, 1997). *RGT1* was disrupted in CMY1005 (*grr1Δ*). Plasmid pSH2-1/MAL63, a *HIS3* vector carrying a *LexA/MAL63* fusion gene under the control of the *ADH1* promoter, was transformed into strain CMY1010 (*grr1Δ rgt1Δ*). Cells were grown in rich media or synthetic media minus histidine with glycerol/lactate plus 2% of specified sugar for maltase assay. ND, not determined.

RGT2-1 (Table 15). Similar results were obtained in strain YPH500 carrying a *MAL63* gene on a *CEN* plasmid (Table 15). Disruption of *RGT2* gene in CMY1001 has little effect on glucose repression of maltase expression in the presence of glucose or glucose plus maltose (Table 14). Therefore, *RGT2* does not appear to be responsible for triggering the Grr1p-dependent glucose inhibition of the *MAL*-activator.

TABLE 15

Effect of an *RGT2-1* allele on maltase expression

MAL-activator allele	Relevant genotype	Maltase activity –	 Mal
MAL13	*RGT2*	27	832
	RGT2 (p*RGT2-1*)	24	562
MAL63	*RGT2*	26	742
	RGT2 (p*RGT2-1*)	30	410

Plasmid pBM3270 carrying an *RGT2-1* allele (obtained from M. Johnston) was transformed into strain CMY1001 containing *MAL13* or strain YPH500 carrying a *MAL63* plasmid. Cells were grown in rich media or synthetic media minus uracil or uracil and tryptophan plus glycerol/lactate with or without 2% maltose. Maltase activities were assayed as described in Table 10.

DISCUSSION

Glucose repression is a multi-faceted regulatory system in *Sacchromyces cerevisiae*. In this report, evidence is presented that defines several components contributing to glucose repression of maltose fermentation. One major pathway is the Mig1p-mediated transcriptional repression of the *MAL* structural genes as well as some of the *MAL*-activator genes. Additional Mig1p-independent mechanisms are identified and include repressing effects mediated by Hxk2p and, more importantly, the glucose inhibition of *MAL*-activator-mediated maltose induction process.

Mig1p is a glucose-responsive repressor of the *MAL* genes. *MIG1* encodes a protein with two C_2H_2 zinc finger motifs that functions as a repressor downstream of the Snf1 protein kinase pathway (Nehlin and Ronne, 1990; Vallier and Carlson, 1994). Mig1p-binding sites were identified in the shared, bidirectional promoter sequence of *MAL61* and *MAL62* at basepairs -273 to -288, called site B, and -578 to -592, called site D. The nucleotide numbers indicate the distance from the *MAL61* ORF. Site D begins 282 basepairs upstream of the *MAL62* ORF and is immediately adjacent to the UAS_{MAL}, a Mal63p binding site (Levine *et al.*, 1992; Ni and Needleman, 1990; Yao *et al.*, 1994). Our results indicate that site D contributes significantly to the glucose-repression of *MAL62* while site B (proximal to the *MAL61*) is primarily responsible for the repression of *MAL61*. In the absence of the proximal binding site, the distal site also seems to have a modest effect on Mig1p-dependent repression as evidenced by the slight relief of glucose repression in the double-site deletions compared to the single deletion of the proximal sites, implying some degree of bidirectionality (Table 4 and 5). Strong evidence for the bidirectional function of Mig1p-binding sites is found in the *GAL* system. A single Mig1p-binding site is found in the shared, bidirectional *GAL1-GAL10* promoter, referred as element A of the upstream repressing sequence, URS_G (Flick and Johnston, 1992; Nehlin *et al.*, 1991). The URS_G A and C elements are located between the UAS_G and the ATG codon of *GAL1* and appear to function independent of orientation upon a heterologous promoter (Flick and Johnston, 1992). No URS_G was found between the UAS_G and the ATG codon of *GAL10* (Flick and Johnston, 1992) and, while *GAL10* expression is clearly sensitive to glucose repression, the role of the URS_G A element in Mig1p-dependent glucose repression of *GAL10* was not tested directly.

Deletion of site D reduced *MAL62-LacZ* expression in the uninduced and induced conditions, and this effect was independent of Mig1p. This result suggests that site D may also bind a transcriptional activator. Potential candidates are the products of *MSN2* and/or *MSN4* genes which were isolated as multicopy suppressors of a *snf1-ts* mutation (Estruch and Carlson, 1993). Msn2p and Msn4p are closely related zinc finger proteins whose DNA-binding domains are somewhat similar to the Mig1p DNA-binding domain and have been suggested to recognize the region bound by Mig1p in the *SUC2* promoter

(Estruch and Carlson, 1993). However, Msn2p and Msn4p differ from Mig1p in position 18 of the second finger which determines DNA-binding specificity (Ronne, 1995). This suggests that their binding specificity probably differs from that of Mig1p, but does not rule out the possibility that the three proteins may bind to partially overlapping sites. Msn2p has recently been identified as a C_4T-binding transcriptional activator involved in yeast multistress response (Schmitt and McEntee, 1996).

Disruption of *MIG1* in *MAL6* strain 332-5A surprisingly results in higher maltase levels in repressed cells than in uninduced cells. This apparent glucose induced effect appears to be specific to strain 332-5A, the basis of which is unclear. It is noteworthy that the constitutive expression of the *MAL* structural genes in the *MAL64-R10* strain is slightly repressed by maltose and this repression effect is dependent upon Mig1p. We have found that maltose also causes inactivation of maltose permease and this inactivation requires the presence of a maltase gene (Jiang, 1997). Thus, the maltose repression effects seen in the constitutive strain may well be the result of maltose hydrolysis catalyzed by maltase which produces glucose.

Sequence-specific binding of Mig1p. The sequences of the Mig1p-binding sites identified in the *MAL* promoter regions are in excellent agreement with previously determined rules for Mig1p binding (Lundin *et al.*, 1994). High-affinity binding of Mig1p requires a GC-box, (G/C)(C/T)GG(G/A)G, with a G being preferred in position 5. In addition, an AT-rich region 5' to this GC-box is also important for binding. The three oligonucleotides that clearly bound Mig1p (JN66, JN72, and JN96) all have a GC-box with a G in position 5, and a well conserved AT-box (Figure 6). JN90, which bound Mig1p very weakly, has both a GC-box and an AT-box, but the GC-box has an A in position 5. In contrast, the oligonucleotide JN94 that failed to bind Mig1p lacks the AT-box. Moreover, it has an A in position 5 of the GC-box.

Dual level control of the *MAL* genes by Mig1p. A Mig1p-binding site was identified in the sequence -116 to -131 basepairs upstream of the *MAL63* ORF. Deletion of *MIG1* relieves glucose-repression of both *MAL63* mRNAs (Figure 4). It has not been determined if both transcripts are functional but, in studies of a strain carrying the *MAL1* locus and no others, only the smaller 1.6 kb transcript was detected suggesting that the smaller *MAL63* transcript, if not both, is functional (Charron *et al.*, 1986). Thus, Mig1p regulates the transcription of the *MAL* structural genes not only directly but indirectly by regulating the transcription of the *MAL*-activator gene.

This dual level control of the *MAL6* genes by Mig1p is similar to its role in repression of the *GAL* genes (Griggs and Johnston 1991; Nehlin *et al.*, 1991), and also to the repression of the *alc* genes by CREA, the Mig1p homologue in *Aspergillus nidulans* (Mathieu and Felenbok, 1994). It therefore seems to be a general finding that glucose repression acts both on specific activators and on their respective target genes. One cannot rule out that other genes such as *SUC2*, which are directly repressed by Mig1p, also may have specific activators whose expression is similarly repressed. Direct repression of the *GAL1* promoter is very fast, and the dual-level control is therefore thought to be important during the early stages of repression (Johnston *et al.*, 1994).

Glucose repression of *GAL4* is 5-fold at the mRNA level, and is completely dependent on Mig1p (Nehlin *et al.* 1991). Our results suggest that repression of *MAL63* is of a similar magnitude (Figure 4). However, the situation is complicated by the presence of two *MAL63* transcripts, and also by the fact that *MAL63*, unlike *GAL4*, is autoregulated. The system is therefore more similar to the *alc* genes in *Aspergillus*, where the specific activator gene *alcR* is regulated both by CREA and by its own gene product (Mathieu and Felenbok, 1994). A Mig1p-independent glucose repression of *MAL63* is still seen in the presence of maltose (Figure 4). Since *MAL63* is autoregulated, it seems likely that this repression simply reflects a glucose-dependent inhibition of the induction pathway.

Interestingly, the Mig1p-binding site present in the *MAL63* promoter is not found at a comparable position upstream of *MAL23* and *MAL43-C* (Gibson *et al.*, 1997). Approximately, 200 basepairs upstream of both genes has been sequenced and the three promoters are 98% identical overall in this region (Gibson *et al.*, 1997). The sequence of the *MAL63* Mig1p-binding site differs by a single basepair from the comparable sites in the *MAL23* and *MAL43-C* promoters, respectively, which lack one G/C basepair in the critical GC-box. The constitutive *MAL43-C* and *MAL23* mutants which lack Mig1p-binding site in their promoters exhibit resistance to glucose repression (Charron and Michels, 1987; Gibson *et al.*, 1997; see also Table 3), suggestive of the abilities of the transcriptionally active *MAL*-activators to overcome at least partially the glucose repression effects on the bidirectional promoter of the *MAL* structural genes. On the other hand, a constitutive *MAL63/43* hybrid gene under the control of the *MAL63* promoter containing a Mig1p-binding site is sensitive to glucose repression. Deletion of *MIG1* significantly relieves glucose sensitivity of *MAL63/43-C* in activating maltase expression as compared to *MAL63*, and only slightly derepresses maltase expression in *MAL43-C* and *MAL64-R10* strains in the presence of glucose. Thus, the repressing effect of Mig1p appears more effective on the *MAL63* promoter than on the promoter of the *MAL* structural genes in counteracting the activation by the *MAL*-activator. Consistent with this view is the report that deletion of the Mig1p-binding site in the *MAL63* promoter relieves glucose sensitivity of the constitutive *MAL63* mutants (Wang and Needleman, 1996), a phenotype resembling *MAL43-C*. Significant repression by Mig1p of *MAL63/43-C* transcription thus appears to be a rate-limiting step leading to an apparent loss of activation of maltase expression in the presence of glucose.

Downregulation of the induction process contributes to glucose repression of the *MAL* genes. *MAL* gene expression in glucose-repressed conditions has been determined both with and without maltose present in the medium. Northern analysis of maltase transcript suggests that maltose induction is inhibited 25-fold by glucose in *MAL6* strain 332-5A and that this inhibition is Mig1p-independent. The *MAL64-R10* and *MAL43-C* *MAL*-activator mutant alleles are fully constitutive and have enabled us to examine the contribution of the induction-dependent component of glucose regulation of *MAL* gene expression in addition to the Mig1p-dependent component. In strains carrying these alleles, expression of the *MAL* genes shows less than 10-fold sensitivity to glucose repression as compared to the 700-fold repression seen in the isogenic inducible strain (Tables 2 and 3). Disruption of *MIG1* in the constitutive strains further relieves glucose repression of *MAL62* by 2 to 3-fold (Table 3, Table 6) and *MAL61* by 5-fold (Table 6). In the *mig1Δ* background, the *MAL*-activator constitutive strains produce significantly higher levels of expression of the *MAL* structural genes than the inducible strain under repressed/induced conditions. These studies indicate that glucose inhibits some aspects of the *MAL*-activator-dependent induction process and this inhibition is responsible in large part for the Mig1p-independent glucose repression.

Glucose may inhibit maltose induction in several possible ways. It could affect the expression of the *MAL*-activator protein; it could cause inducer exclusion; it could block the maltose signal transduction pathway; and it could affect the posttranslational modification of the *MAL*-activator or alter the protein-protein interactions between the *MAL*-activator and other regulatory factors.

Glucose inhibits the *MAL*-activator function by an induction-dependent mechanism. Although mRNA levels of *MAL63* in *mig1Δ* cells grown in glucose or glucose/maltose media are increased to about half that of maltose-induced levels, Mal63p-dependent maltose induction is still inhibited by glucose which raises the possibility that glucose could affect the synthesis and/or stability of the Mal63 protein. Our results show that such down-regulation in glucose media, while perhaps sufficient, apparently is not essential for glucose inhibition of the Mal63p function. Overexpression of *LexA-MAL63* in a *mig1Δ* strain fails to overcome the inhibitory effect of glucose on its ability to activate both the maltase and the *lexA-LacZ* reporter genes. It is unlikely that the ability of the overexpressed LexA-Mal63 fusion protein to bind to DNA is directly affected by glucose. We found no evidence that the LexA-MAL63 fusion protein fails to bind to lexA operator sites in the promoter region of the *LacZ* reporter gene in response to glucose. A similar fusion in which *MAL63* is fused to full length $LexA_{(1\text{-}202)}$, was shown to bind to the lexA operators regardless of carbon sources (unpublished data). In addition, it was found that high dosage of Mal63p is able to compete with Mig1p binding since these two binding sites are located next to each other in the *MAL61-MAL62* promoter suggesting that overexpressed Mal63p is able to bind to UAS_{MAL}, especially when *MIG1* is deleted (Wang and Needleman, 1997). Thus, we favor the view that a function of Mal63p other than DNA-binding is inhibited in the presence of glucose.

MAL-activator-mediated maltose induction requires the presence of a functional maltose permease gene (Charron *et al.*, 1986; Dubin, 1987). It is generally believed that the role of maltose permease in induction is to transport the inducer, maltose, into the cell and that glucose-induced inactivation of maltose permease eliminates the transport of the inducer thereby preventing the induction process, a phenomena termed "inducer exclusion". Disruption of *HXK2* which derepresses transcription of maltose permease gene but maintains glucose-induced inactivation of maltose permease protein relieves glucose inhibition of maltose induction, indicating that inducer exclusion caused by glucose-induced inactivation of maltose permease alone is not sufficient to block the induction. This result suggests that inducer exclusion may not be a major player in glucose inhibition of maltose induction. We show that the expression level of maltose permease required for induction, as measured by maltose transport activity, is remarkably low. Several *LexA-MAL63* mutant fusions in which the N-terminal DNA-binding motif or adjacent regions of Mal63p are deleted, including *LexA-MAL63*$_{(\Delta 41\text{-}66)}$, fail to activate the expression of the *MAL* structural genes but still activate the transcription of the *lexA-LacZ* reporter even with the extremely low basal level of maltose transport activity. Moreover, raising maltose permease activity significantly above the basal level is unable to relieve the inhibition of the LexA-Mal63$_{(\Delta 41\text{-}66)}$ activity by glucose. Thus, elimination of inducer exclusion is not sufficient to alleviate glucose inhibition. Nevertheless, given the low level requirement of maltose permease expression for induction, we can not exclude the possibility that the combination of both glucose repression of maltose permease transcription and glucose-induced inactivation of maltose permease protein acts synergistically to functionally eliminate maltose permease in cells grown on glucose plus maltose, thereby creating a non-inducible phenotype resembling that of maltose permease gene disruption strain.

The fact that the uninduced basal level of maltose permease activity is adequate for induction may imply a regulatory role for maltose permease in the induction process. In support of this hypothesis, cells with a deletion of the maltose permease gene are unable to produce induced level of maltase in the presence of as high as 15% maltose despite the fact that these cells are able to take up sufficient maltose to grow at a slow rate using basal level of maltase for maltose hydrolysis (unpublished data). Maltose permease contains a relatively long carboxyl-terminal tail located in the cytoplasmic face of the plasma membrane, analogous to glucose sensors Snf3p and Rgt2p. In this regard, we can not exclude the possibility that glucose may effectively inhibit the regulatory function of maltose permease rather than its ability to transport maltose.

In the absence of inducer exclusion and down-regulation of Mal63 protein levels as major factors contributing to glucose repression of *MAL* gene expression, we conclude that glucose inhibits the maltose induction pathway and/or events required for the *MAL*-activator-mediated induction process, which we called "induction-dependent mechanism". Transcription activation by Gal4p has been found to be directly inhibited by glucose through the central region of Gal4p protein (Stone and Sadowski, 1993). This inhibition does not appear to alter the ability of Gal4p to bind to DNA (Huibregtse *et al.*, 1993). Similarly, our findings indicate that it is unlikely that the N-terminal half of the *MAL*-activator containing the DNA-binding domain and activation domain is a direct target of glucose inhibition. The constitutive *MAL64-R10* contains a nonsense mutation at codon 283 generating a truncated activator protein (Gibson *et al.*, 1997). A *LexA-MAL63*$_{(1\text{-}283)}$ fusion in which the N-terminal 283 codons of *MAL63* are fused to *LexA* under the control of an *ADH1* promoter also is constitutive (Gibson, 1995). These two constitutive activators lacking the C-terminal maltose-regulatory domain but possessing the DNA-binding and activation domains both are insensitive to glucose inhibition (Table 3; unpublished results). Furthermore, the full-length constitutive mutants of *MAL23* and *MAL43*, as well as those of *MAL63* when their expression is not repressed by Mig1p, all become resistant to glucose inhibition (Gibson *et al.*, 1997; Wang and Needleman, 1996). These evidences all suggest a correlation between maltose inducibility and glucose inhibition sensitivity.

The C-terminal portion of various *MAL*-activators contains a maltose regulatory domain (Gibson *et al.*, 1997) and thus may be involved in glucose inhibition. The complete glucose sensitivity of strains carrying wild-type *MAL13* or *MAL33* (Table 11; unpublished data) indicates that these alleles are subject to glucose inhibition of maltose induction. It therefore appears common to these *MAL*-activators that their function as maltose-inducible transcription activators is inhibited by glucose. However, strains carrying wild-type *MAL23* surprisingly display partial maltase expression in maltose/glucose media. On the other hand, an inducible *MAL63/23* hybrid gene, in which the C-terminal 255 residues (codon 216-470) of Mal23p containing most of the variations compared to Mal63p is fused to the N-terminal 215 residues of Mal63p, is completely sensitive to glucose in a *mig1Δ* strain. The C-terminal maltose-responsive domain of Mal23p is thus not sufficient to account for its partial resistance to glucose inhibition. Glucose insensitivity of the truncated constitutive mutants, such as *MAL64-R10* and *LexA-MAL63*$_{(1-283)}$, suggests that the DNA-binding and transcriptional activation functions, both of which are located in the N-terminal portion of the *MAL*-activators, including Mal23p, are not the targets of glucose inhibition. Thus, the partial insensitivity of *MAL23* may imply a genetic interaction between N-terminal and C-terminal parts of the *MAL*-activator protein involved in maltose induction and glucose inhibition.

***GRR1* is required for glucose inhibition of maltose induction but independent of the roles of *RGT1* and *RGT2*.** Effects of several genes which are involved in glucose repression of *SUC2* and *GAL* genes and glucose induction of *HXT* genes were examined in order to analyze the glucose regulatory pathway responsible for inhibition of maltose induction. Glucose induced expression of *HXT1* is mediated by high-glucose sensor Rgt2p and dependent upon Grr1p (Ozcan *et al.*, 1996). Our data indicate that Grr1p is required for glucose inhibition of the *MAL*-activator function in maltose/glucose-grown cells. However, this Grr1p-dependent pathway does not appear to involve Rgt2p and, thus, differs from the glucose induction pathway responsible for *HXT* expression.

Disruption of *GRR1* also relieves glucose repression of maltase expression in glucose-grown cells. The glucose repression defect of *grr1Δ* mutants is suppressed by disruption of *RGT1* (Table 14) which is a repressor of *HXT* transcription and subject to a Grr1p-dependent inactivation in response to glucose (Ozcan and Johnston, 1996; Ozcan *et al.*, 1996). The correlation between restored glucose transport and restored glucose repression of maltase expression in *grr1Δ rgt1Δ* double mutants is consistent with the idea that high-affinity glucose transport is required for normal glucose repression. As a result of restored normal glucose repression in *grr1Δ rgt1Δ* mutants, maltase expression is accordingly reduced in the presence of maltose plus glucose. However, disruption of *RGT1* in *grr1Δ* strain is unable to restore glucose inhibition of the *MAL*-activator function, as evidenced by the partial induction of maltase expression in maltose/glucose medium (Table 14). This conclusion is further supported by the observation that in *grr1Δ rgt1Δ* cells carrying the overexpressed *LexA-MAL63* fusion gene, maltase expression is apparently induced in maltose/glucose medium. We conclude from these results that the role of Grr1p in mediating glucose inhibition of the *MAL*-activator function is in addition to and distinct from its role in the inactivation of the Rgt1 repressor.

Roles of *HXK2* in glucose repression of *MAL* gene expression. *HXK2* encoding hexokinase PII has been proposed to act at an early step of glucose signal transduction pathways (Johnston and Carlson, 1992; Ozcan and Johnston, 1995). Deletion of *HXK2* is sufficient to abolish glucose inhibition of the *MAL*-activator function in maltose/glucose-grown cells as long as the *MAL*-activator gene transcription is not repressed by glucose. The LexA-Mal63 fusion protein is able to activate expression of both maltase and the *LacZ* reporter genes without *HXK2* in the presence of maltose plus glucose. It appears that the role of Hxk2p in mediating glucose inhibition of the *MAL*-activator is unique among the glucose-phosphorylating enzymes under the conditions examined.

Disruption of *HXK2* relieves glucose repression in cells grown in glucose media by a mechanism that appears to be independent of Mig1p. The ability of Mig1p to repress *MAL* gene expression in an *hxk2Δ* background is somewhat unexpected given the notion that Hxk2p functions upstream of Mig1p. Yeast contains three glucose-phosphorylating enzymes encoded by *HXK1*, *HXK2*, and *GLK1*. Noting that the expression of *HXK1* (encoding hexokinase PI) and *GLK1* (encoding glucokinase) is derepressed in glucose media in *hxk2Δ* background (De Winde *et al.*, 1996), it is perhaps not surprising that *HXK1* actually mediates the residual glucose repression of *MAL* gene expression observed in the absence of *HXK2*. Disruption of *GLK1* in *hxk2Δ* background also relieves glucose repression further, although the effect is less pronounced than seen for disruption of *HXK1* (data not shown). Therefore, *HXK1* and to a lesser extent, *GLK1*, can at least partially substitute for *HXK2* in mediating regular glucose repression when their expression is not repressed by glucose in *hxk2Δ* background. We infer from these findings that *HXK1* is responsible for generating and/or transmitting a glucose signal that results in the Mig1p repression observed in *hxk2Δ* background. Mutation of *HXK2* was found to have only a slight effect on URS_G-mediated glucose repression of *GAL1* (Flick and Johnston, 1990). Perhaps *HXK1* also partially mediates glucose repression of *GAL1* in a *hxk2* strain.

The redundant roles played by various glucose-phosphorylating enzymes support the idea that glucose phosphorylation is essential for generating/transmitting the glucose signal. Studies using strains expressing only one of the three glucose-phosphorylating enzymes showed that *HXK1* and *GLK1* are both able to mediate glucose-induced inactivation of maltose permease, though to lesser extents than *HXK2* (Jiang, 1997). These two enzymes acting together may account for the glucose inactivation of maltose permease observed in a *hxk2Δ* strain which is identical to that seen in the wild type strain. However, Hxk1p and Glk1p are unable to confer glucose sensitivity of the *MAL*-activator through the induction-dependent mechanism in the same *hxk2Δ* strain. This result may suggest an effect of the different expression levels of the glucose-phosphorylating enzymes on their roles in mediating glucose inhibition of maltose induction.

***REG1* is involved in the induction-dependent mechanism and negatively regulates Mal63p activity.** *REG1* encodes a glucose repression responsive regulatory subunit of protein phosphatase type-1 (Tu and Carlson, 1995). It was reported that maltose has inhibitory effects on *reg1* mutants carrying a *MAL2-8^c^* gene which appear to result from intracellular accumulation of glucose (Entian and Loureiro-dias, 1990). We have found that the inhibitory effect of maltose on *reg1Δ* cells appears to be *MAL*-activator allele-dependent (Jiang H., unpublished data). Transformation of *MAL63* into a *reg1Δ* strain, DF186, causes no growth defect on maltose, thereby allowing us to examine the effects of *REG1* on glucose inhibition of Mal63p-mediated maltose induction.

Disruption of *REG1* relieves Mig1p-dependent glucose repression of *MAL* gene expression, consistent with the model in which Reg1p acts upstream of Mig1p in glucose repression regulatory pathway. In addition, *REG1* is required for the induction-dependent mechanism to inhibit the *MAL*-activator function in response to glucose. Overexpression of *REG1* significantly reduces *GAL* and *SUC2* expression in the absence of glucose (Niederacher and Entian, 1991; Tu and Carlson, 1995). Multiple copies of *REG1* are found to only slightly affect maltose-induced expression of maltase (Jiang, 1997), suggesting that the Reg1p-dependent mechanism responsible for glucose inhibition of the Mal63p function may differ from that mediating glucose repression of these other genes.

Mechanism of glucose inhibition of maltose induction. Our results indicate that *HXK2*, *GRR1*, and *REG1* are required for glucose inhibition of *MAL*-activator function in addition to Mig1p-dependent glucose repression of *MAL* gene expression. *HXK2* and *REG1* appear to be required for the Rgt1p-independent glucose induction of *HXT1* (Ozcan and Johnston, 1995). *REG1* appears to be involved in *RGT2-GRR1* pathway mediating glucose-induced inactivation of maltose permease (Jiang *et al.*, 1997; Jiang, 1997). Various glucose-responsive pathways therefore seem to share some of the primary components but the exact roles of these factors in various pathways and whether they function the same way in differently regulated systems has yet to be determined.

The induction-dependent mechanism could affect the modification of the *MAL*-activator essential for its function. It may regulate the expression and/or activities of other unidentified factors that are involved in maltose signal transduction pathway or required for induction of the *MAL*-activator. Hxk2p is most likely responsible for generating and/or transmitting a glucose signal to inhibit the *MAL*-activator function. The fact that other glucose-phosphorylating enzymes are able to substitute for Hxk2p in mediating normal glucose repression but not glucose inhibition of maltose induction suggests that Hxk2p performs multiple regulatory functions some of which may be specific and essential to the *MAL* genes. It has been shown that *GRR1* is required for degradation of the G1 cyclins Cln1 and Cln2 and one of the defects associated with *grr1* mutants, abnormally elongated cell morphology, is the result of G1 cyclin over-accumulation (Barral *et al.*, 1995). Grr1p appears to be a E3 component of the ubiquitin-conjugating enzyme complex and recruits various specific targets for degradation (Li and Johnston, 1997). It is thus possible that an unidentified protein involved in the maltose signaling pathway or required for proper function of the *MAL*-activator is a direct target of Grr1p in the presence of glucose. Such a protein could be responsible for posttranslational modification of the *MAL*-

activator that is essential for its function. Alternatively, it could be required for generating and/or transmitting the maltose signal. We do not believe that the *MAL*-activator is a direct target of Grr1p since overexpression of the *MAL*-activator is unable to abolish glucose inhibition. Reg1p is proposed to direct the Glc7p phosphatase to specific substrates involved in glucose regulatory pathway (Tu and Carlson, 1995). One possible target of Reg1p/Glc7p could be a component of maltose induction pathway. It is equally possible that a regulator of Reg1p is a target of Grr1p or vice versa in the glucose signal transduction pathway. Characterization of the maltose induction process will provide valuable insight as to which component is directly subject to glucose inhibition.

Role of other factors in regulation of the *MAL* genes. Based on the results of *MIG1* disruption in the inducible *MAL6* strain (Table 2 and Table 4), it was expected that disruption of *MIG1* in the *MAL64-R10* constitutive strain would all but eliminate glucose-repression sensitivity. It did not (Table 3 and Table 6). The persistence of a 2 to 6-fold glucose repression of either *MAL61-LacZ*, *MAL62-LacZ* or maltase expression in constitutive, *mig1Δ* strains indicates that other glucose-responsive regulatory factors also are involved. Disruption of *HXK2* alone also relieves some of the repression effects that appear to be independent of Mig1p (Table 10). It has been shown that *MIG1* acts synergistically with *SSN2* through *SSN5*, *SSN7* and *SSN8* in mediating glucose repression of *SUC2* (Vallier and Carlson 1994). Conceivably, these *SSN* genes could also be involved in glucose repression of the *MAL* genes.

In summary, we have uncovered several pathways involved in the glucose regulation of *MAL* gene expression (Figure 8). One major pathway is the Mig1p-mediated repression of *MAL* gene transcription, including that of *MAL63*. The other, even more significant pathway for repression, is seen only under induced/repressed conditions and represents the inhibition of the *MAL*-activator-mediated maltose induction in response to glucose availability. Hxk2p, a glucose-phosphorylating enzyme, may be responsible for generating/transmitting a signal that controls both Mig1p-dependent and Mig1p-independent glucose repression. Reg1p is involved in both Mig1p-dependent glucose repression and Mig1p-independent glucose inhibition of maltose induction but may perform different regulatory functions in each pathway. In addition to being involved in glucose induction of *HXT* gene expression which is required for glucose repression, Grr1p is essential for and plays a distinct role in glucose inhibition of maltose induction. The Mig1p-dependent glucose repression and Hxk2p-dependent mechanisms account for almost all the glucose repression effects observed in the strains used throughout this study.

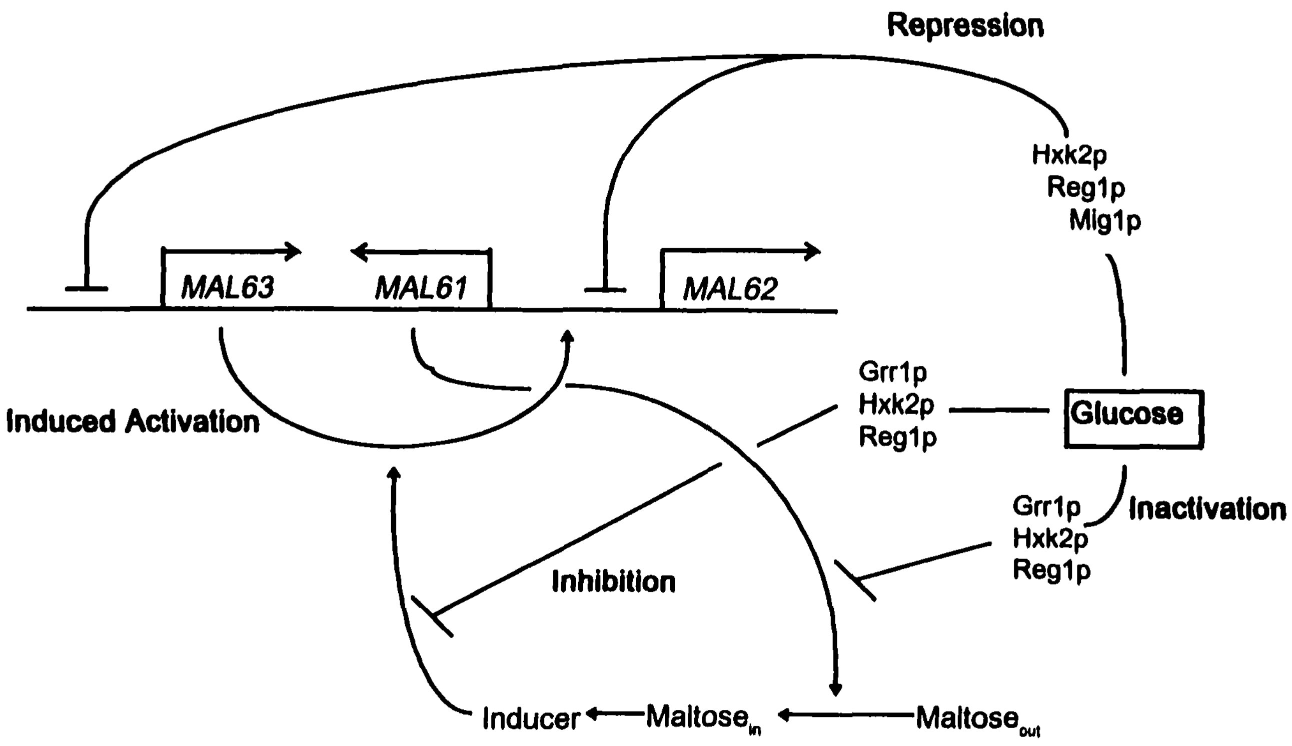


Figure 8. Model of glucose regulation of *MAL6* gene expression.

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